Chapter

Phage Display as a Strategy to Obtain Anti-flavivirus Monoclonal Antibodies

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Abstract

Arbovirus of the *Flaviviridae* family represents an issue worldwide, particularly because it can lead to serious illness and death in some countries. There is still a great complexity in obtaining effective therapies and specific and sensitive diagnostic tests, due to the high antigenic similarity between them. This similarity may account for antibodies cross reactivity which has positive and negative consequences for the course of infectious diseases. Among dengue virus (DENV) serotype infections, the cross-reactivity can increase virus replication and the risk of a severe disease by a mechanism known as an antibody-dependent enhancement (ADE). The search for serological biomarkers through monoclonal antibodies (MAbs) that identify unique viral regions can assist in the differential detection, whereas the development of recombinant antibodies with a neutralizing potential can lead to the establishment of efficacious treatments. The Phage Display methodology emerged as one of the main alternatives for the selection of human MAbs with high affinity for a specific target. Therefore, this technology can be a faster alternative for the development of specific diagnostic platforms and efficient and safe treatments for flavivirus infections. In this context, we propose for this chapter a discussion about Phage Display as a strategy to obtain MAbs for DENV and other flaviviruses.

Keywords: antibody, Phage Display, dengue virus, flavivirus, therapy, diagnosis

1. Introduction

When thinking about the development of virus detection and neutralization technologies whose bases of action are immunoglobulins, it is necessary to understand the structure of the viral particle of interest. In addition to the sequence of amino acid residues that make up the target epitopes, their position in the particle and their function in the process of infection and viral replication influence the design experiments aiming the obtantion of antibodies with a diagnostic and therapeutic potential.

The structures of flavivirus have been determined and studied, mainly, by combining cryo-electron microscopy with data from X-ray diffraction experiments

using crystallography of viral proteins in the presence or not of antibody molecules. Results of this combination showed that the flavivirus is composed of a dense icosahedral electron nucleus and a lipid bilayer surrounding it. The genome comprises a sequence of ~10,700 nucleotides of a positive-sense RNA that encodes a polyprotein that is cleaved in 3 structural proteins, capsid protein (C), membrane protein (M), and envelope protein (E), and in 7 nonstructural proteins, NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 [1]. The NS1 protein plays a role in viral replication and is often shown to be a soluble antigen secreted into the bloodstream, interacting with components of the immune system. NS5 is the largest and most conserved nonstructural protein and acts in the transcription process of viral RNA [2, 3].

The icosahedral capsid is small and poorly organized, but it provides enough space for the genome and is surrounded by the envelope, so there are few connections between these structures, unlike what is found in other viruses. The viral envelope consists of two internal and concentric layers of phospholipids and an outer protein shell formed by regions of protein M and ectodomains of glycoprotein E organized in dimers which present protein determinants for the binding of the virus to the host cell (hemagglutination). Protein E contains three domains: domain I (DI), which is related to cell tropism and envelope organization; domain II (DII), which comprises the dimerization region and the fusion peptide; and domain III (DIII) with the function of binding to cell receptors, in the initial stage of viral infection [4, 5].

The fusion loop is a highly conserved region between dengue virus (DENV) serotypes and all flaviviruses, responsible for the late stage of infection, in which the virus interacts with the endosomal membrane, resulting in the release of the nucleocapsid in the cytoplasm. When the particle is in the lysosomal vesicle during the infection process, a structural reorganization of the viral envelope occurs; the fusion loop is exposed and inserted in the lysosomal membrane. After the formation of the fusion loop contact, protein E starts to organize itself into trimmers, resulting in the expansion of the viral particle and the approximation of the viral and lysosomal membranes, forming the fusion lipid pore that allows the release of the viral genome to the cytoplasm [3, 6, 7].

DENV serotypes show great heterogeneity in the structure of viral proteins. However, there is also antigenic similarity between DENV serotypes and, for some peptide sequences, between flaviviruses. Phylogeny studies of virus sequences, by estimating the antigenic distance between them, concluded that serotypes 1 and 3 are the most similar, serotype 2 was the second to diverge evolutionarily, and serotype 4 is the one that presents greater genetic difference. There is a 32% difference in the structure of protein E of the four DENV serotypes. Specific mutations in the genome result in the antigenic variability found in each serotype [7–9].

Much of the genetic difference between flaviviruses is due to protein E, which can show up to 60% difference in its amino acid sequence. In the phylogenetic analysis of the viruses, the DENV serotypes are closer to the Zika virus (ZIKV), with approximately 45% difference, and have 50 and 60% dissimilarity with West Nile virus (WNV) and yellow fever virus (YFV), respectively. DENV and other flaviviruses vary dramatically in terms of the amino acid sequence of the glycosylation region and the content of glycans added to the surface of E and precursor membrane protein (prM). Many epitopes of protein E are unique to a DENV serotype [4, 9–11]. It can also be observed in ZIKV, which has the glycosylation site, in the DI of protein E, different in conformation and length of the loop that contains this glycosylation site. The carbohydrate associated with this residue can act as a virus binding site in host cells. Thus, differences in this region of glycosylation can influence cell tropism, infection, and pathogenesis of these viruses [12]. Another important characteristic of ZIKV is the insertion of an alanine residue in the carboxyl termination of DIII, which is associated with an increased stability of this virus [13].

The hydrophobic sequence of the fusion loop appears to be the only epitope that is conserved among all flaviviruses; however, the degree of exposure in this region varies substantially among viruses [6]. Nonstructural proteins, NS1 and NS5, also present some epitopes conserved among the DENV serotypes and other members of the flavivirus genus, but their position also varies between viral strains. The relationships of antigenic similarity between flaviviruses generate immune responses that are configured as cross-reactions with protective or pathological characteristics [5, 14, 15].

2. Immunoglobulins

Immunoglobulins, or antibodies, are glycoproteins, expressed on the surface of B cells or secreted, that act in the neutralization and elimination of pathogens [16]. Antibodies are relatively flexible "Y"-shaped molecules made up of two heavy chains and two light chains, joined by an extensive network of non-covalent interactions, stabilized by disulfide bonds. Both types of chains are composed of constant and variable domains. The constant regions determine the functional properties of the antibody, and the variable regions determine the antigen-binding site. The light chain consists of a variable portion (VL) and a constant portion (CL) that can have two types of domains, kappa (ƙ) or lambda (λ). The heavy chain consists of a variable portion (VH) and three or four constant portions, depending on the class of the antibody, which are CH1, CH2, CH3, and CH4. The type of heavy chain determines the class, or isotype, of antibody, such as IgA, IgG, IgD, IgE, and IgM [17].

The antibody molecule can be subdivided into portions of the crystallizable fragment (Fc) and antigen-binding fragment (Fab) region. The Fc portion has the constant domains (CH2, CH3, CH4), and the Fab portion consists of the VH-CH1 and VL-CL domains. The Fab portion retains the ability to bind to the antigen, and the Fc portion acts to mediate the effector functions of antibodies [17].

Three segments containing variability can be identified in both the VH and VL domains. These segments are the hypervariable regions that determine antigen specificity and are more commonly called complementarity-determining regions (CDRs)—CDR1, CDR2, and CDR3. The combination of CDRs from a VH with CDRs from a VL forms the region of interaction with the epitope, called the paratope [17]. The variability of the antigen-binding regions is responsible for the ability of different antibodies to bind to many structurally diverse antigens [18]. **Figure 1** represents the structure of an IgG immunoglobulin and its domains.

In cognate antigen recognition, some naïve B cells can initiate somatic hypermutation, generating new variable domains, that can be selected based on their ability of antigen binding, usually with high affinity compared to germinal domains. After antigen recognition, naïve B cells differentiate into antibody-secreting plasma cells. These plasma cells secrete antibodies with high affinity and, can differentiate into memory B cells. Memory B cells are highly specialized cells for quickly recognizing the antigen in subsequent exposure, can persist for years, and provide long-term humoral protection for decades. These functional features of memory B cells are the basis of effective vaccines [19].

The knowledge about the mechanisms of antibody production and clonal selection of B cells led to the development of innovative hybridoma technology in 1975 [20]. The technique is based on the fusion of B lymphocytes with myeloma cells giving rise to hybrid cells that produce monoclonal antibodies (MAbs) continuously in vitro [21]. Therefore, MAbs are monovalent antibodies, which bind to the same epitope and are produced from a single B lymphocyte clone.

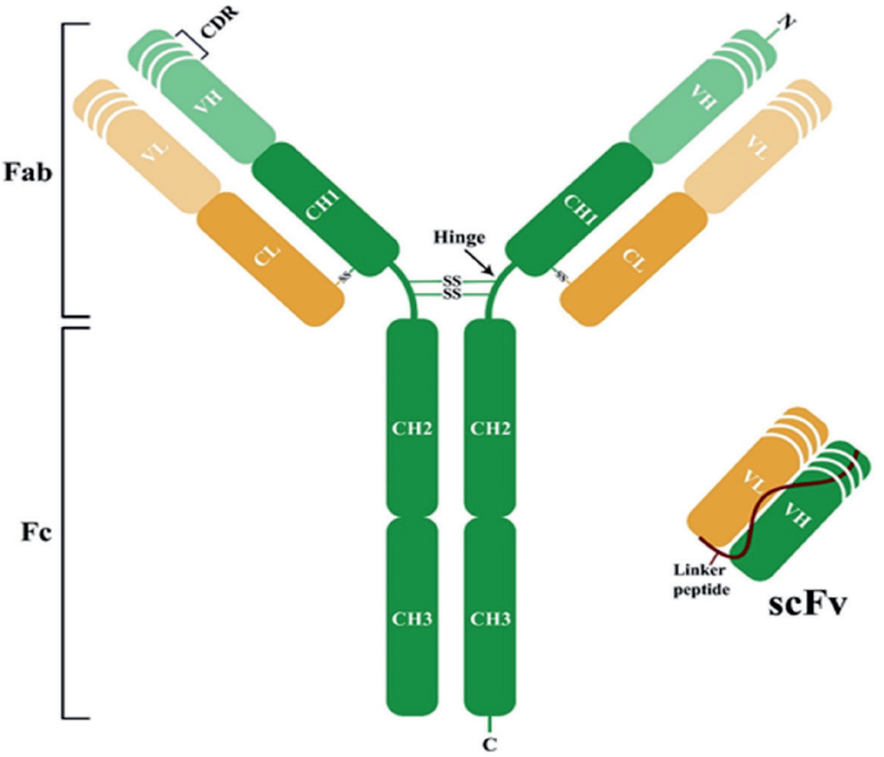

Figure 1.
Classical structure of an antibody. Structure of a class G immunoglobulin, representing the two portions of the molecule: two Fabs that correspond to the antigen-binding fragment and an Fc that corresponds to the crystallizable fragment. A type G antibody consists of two heavy polypeptide chains, each containing a VH and three constant domains (CH1, CH2, and CH3), and two light chains, each containing a VL and a CL. CDRs are three regions of hypervariability present in each of the variable domains. In addition to the natural format of the antibody, it is possible to generate recombinant antibodies such as the single-chain variable fragment (scFvs).

MAbs interact with a single epitope allowing a specific reactivity and affinity for target antigens. This feature is a great advantage over polyclonal antibodies, which have different epitope specificities and affinities [22, 23]. For this reason, MAbs have a broad clinical applicability in therapy for various illness, including cancer, transplant rejection, and autoimmune, infectious, hematologic, and cardiovascular diseases. Moreover, MAbs can play a significant role in the diagnosis and as antibody-drug conjugate for drug delivery. Thus, MAbs are considered a powerful tool for a wide range of medical applications.

2.1 Cross-reactivity of antibodies

Antibodies that bind to different flaviviruses are able to promote both the neutralization of the infection and the increase of the virus capture, such as by the interaction of immune complexes with Fc receptors expressed in certain cell types. The creation of alternative routes of entry of the viral particle into cells by low-neutralizing antibodies, during secondary infections of flavivirus, results in increased levels of viral replication and pathogenicity. This mechanism constitutes a phenomenon called antibody-dependent enhancement (ADE) [24, 25].

Different studies have been conducted to understand the effects of cross-reactive memory antibodies on subsequent flavivirus exposures. Many of them reported that the opsonization of the virus with weakly neutralizing antibodies led to the increase of the viral production and of pro-inflammatory mediators. This could lead to the suppression of the antiviral immune response, worsening the clinical condition of the disease [5, 7]. ADE has already been reported in in vitro experiments of infection of cells that express Fc receptors and in vivo experiments

of vaccination with flavivirus [11, 26]. From the results of these experiments, ADE is pointed out as one of the main causes of severe forms of DENV infection and of the low protection induced by vaccines targeting DENV serotypes [27].

An opposite effect of cross-reacting antibodies has also been demonstrated, an increase in protection against secondary infections by flavivirus, which has resulted in potent neutralization and rapid induction of affinity maturing immune responses against heterologous flaviviruses. Studies in endemic areas of flavivirus showed protection against Zika virus infection in patients with a previous experience with DENV. A humoral response capable of potentially neutralizing both species of flavivirus was assembled from the expansion of cross-reaction memory B cell clones, even in the absence of DENV circulation. Thus, previous flavivirus infections can lead to both cross-neutralization and increased pathogenicity of the virus through the formation of interspecific antibody memory [28, 29].

The potential of cross-reaction immunity to trigger protection or pathology depends on the profile, quality, and magnitude of the immune responses induced by antibodies. The ADE reaction is a factor that should be considered in the development of therapeutic antibodies and vaccines for infections by flavivirus. Different approaches have been tested to shift the ADE profile to a cross-protection profile in heterogeneous infections of these viruses.

3. Phage Display

The principle of the Phage Display is the presentation of libraries of molecules on the surface of a bacteriophage (phage), allowing the identification of a wide range of biomolecules, including peptides, antibodies, and other proteins. The Phage Display methodology was first described in 1985, by George Smith and colleagues. Through the expression (display) of polypeptides on the phage surface (phage) M13, it was possible to perform the mapping of antibody epitopes by screening them using random peptide libraries [30]. In 1990, McCafferty and colleagues [31] demonstrated that it was also possible to fuse genes that encode an entire antibody domain, in the form of a scFv to the sequence of one of the bacteriophage's coat proteins. This approach allowed that this methodology could also be used for the selection of bacteriophages that recognize antigens.

Later, in 1994, Winter refined the Phage Display technology through a guided selection strategy of human antibody fragments from Phage Display repertoires for a single-antigen epitope, using rodent MAbs as a model [32]. The first all-human antibody produced, using Winter's Phage Display technique, to be marketed for use in humans was adalimumab (Humira), approved by the United States Food and Drug Administration (FDA) in 2002 for the treatment of rheumatoid arthritis [33]. It is noteworthy that George P. Smith and Gregory P. Winter received the Nobel Prize in chemistry in 2018 for the Phage Display of peptides and antibodies (Nobel Prize, 2018), a true tool for molecular evolution *in vitro* emphasizing the importance of this technique in obtaining biomolecules for various applications.

Phages are single-stranded viruses that infect Gram-negative bacteria and are used mainly for the purpose of gene cloning and expression of recombinant proteins, in addition to basic molecular biology studies. The particle coating is composed of five different proteins, pIII, pVI, pVII, pVIII, and pIX; proteins responsible for DNA replication include pII, pV, and pX; and the assembly proteins are pI, pIV, and pXI. All of the five proteins contribute to the stability of the phage particle; however, pIII is also necessary for the recognition and infection of the host cell [33]. Through genetic manipulation, sequences of billions of peptides, protein variants, and antibody fragments can be cloned into a vector associated with the

phage coat protein gene, the pIII protein being the most commonly used [34]. Thus, the Phage Display methodology explores the possibility of direct binding of a certain protein (phenotype) with its cognate gene (genotype) by means of a phage [35].

3.1 Antibody Phage Display

Beyond the Phage Display and hybridoma technique, other strategies used for MAb production include immortalization of human B lymphocyte isolated from naturally infected or immunized individuals. One of the approaches for B lymphocyte immortalization is using Epstein-Barr virus (EBV). EBV is a human tumor virus that was shown to infect efficiently human B lymphocytes and induce continuous proliferation in vitro, opening a new perspective for the production of human MAbs [36]. Another relevant alternative for MAb production involves transgenic animals where mice are genetically manipulated to produce human immunoglobulin. In this strategy, genes of human immunoglobulins are inserted into mice genome replacing the endogenous sequences, making these animals capable to produce fully human antibodies when immunized with an antigen [37].

Among the existing methodologies of antibody production, the hybridoma technique remains the most widely used. However, the production steps are laborious and dependent on the animal immune system. In addition, the heterologous character of these proteins often makes them immunogenic to humans, provoking the response of human anti-mouse antibodies (HAMA), which restrict their therapeutic use [34]. Therefore, the Phage Display has emerged as one of the main alternatives for the generation of human recombinant MAbs. The major advantages of using the Phage Display, in contrast to the hybridoma technique, are clearly the absence of the use of animals in the process and the less time to obtain antibodies. The conventional method requires immunization which, depending on the type of antigen, can take weeks to produce sufficient immune response to produce specific antibodies [38].

There are important advantages and disadvantages between techniques for obtaining human MAbs. With the Phage Display technology, it is possible to isolate antibodies against all types of antigens, even those with high complexity; differently, the immortalization technique of human lymphocytes does not allow the isolation of antibodies against own antigens or non-immunogenic antigens [39]. In addition, only the Phage Display allows the optimization of MAbs, for example, by affinity maturation, and in general, the development of antibodies on the Phage Display tends to be faster than in other methods [40].

In addition to being robust due to the high stability of the phage, the Phage Display also allows control over biochemical parameters throughout the selection process. The particular advantage of having control over biochemical parameters during the time of selection can also be used to shape the specificity profile of an antibody from the start [41]. **Table 1** described the MAbs with FDA approval that was developed using the Phage Display technique.

Since 1990, different antibody formats have been employed in the construction of antibody Phage Display libraries (APDLs). Although antibody libraries are one of the most successful tools of Phage Display, the appropriate choice of antibody library is an important step for the success of antibody selection. Full-length antibodies in the immunoglobulin format are large (150 kDa), complex, and not suitable for Phage Display. Therefore, smaller antigen-binding fragments are used. For this reason, APDLs are in most cases constructed in either scFv (25 kDa), Fab (50 kDa), or single-domain antibody (sdAb) formats which are smaller and more effective, although each antibody format has its own advantages and limitations [42]. Particularly, sdAbs have received a growing interest as a promising antibody

Antibody	Target	Format	Indication	Company	Year
Humira adalimumab	TNF-α	Human	Rheumatoid arthritis and Crohn's disease	Abbott	2002
Lucentis Ranibizumab	VEGF-A	Humanized	Macular degeneration	Genentech	2006
Simponi Golimumab	TNF-α	Human	Rheumatoid arthritis	Johnson & Johnson	2009
Benlysta Belimumab	BLys	Human	Systemic lupus erythematosus	GSK	2011
Pending Raxibacumab	PA	Human	Anthrax infection (*Bacillus anthracis*)	GSK	2012
Cyramza Ramucirumab	VEGFR2	Human	Gastric cancer	Lilly	2014
Bavencio Avelumab	PD-L1	Human	Merkel cell carcinoma	Serono	2017
Tremfya Guselkumab	IL-23	Human	Plaque psoriasis	Janssen Biotech	2017
Gamifant Emapalumab	IFNγ	Human	Hemophagocytic lymphohistiocytosis	Swiss	2018

TNF-α, tumor necrosis factor alpha; VEGF-A, vascular endothelial growth factor A; BLys, B lymphocyte stimulator; PA, protective antigen; VEGFR2, vascular endothelial growth factor receptor 2; PD-L1, programmed death-ligand 1; IL-23, interleukin-23; IFNγ, interferon-γ

Table 1.
Monoclonal antibodies obtained by Phage Display with FDA approval.

class compared with those conventional. Their more hydrophilic structure, easy molecular manipulation, convex surface, and long CDRs enable them to recognize cryptic and inaccessible epitopes for typical antibody fragments [43].

There are many kinds of APDLs; they can be classified into two main types: natural APDL and synthetic APDL. This classification is based on the source of VH and VL genes. Natural APDL comprises immune libraries and naïve libraries, while synthetic APDLs comprise semisynthetic libraries and fully synthetic libraries [44]. The immune libraries use V-genes that already passed to the clonal selection and encode antibodies with high affinity and specificity against the target antigen. The immune APDLs have some advantages compared to other libraries, once they have the possibility to be explored for understanding the humoral responses in the specific disease. However, some limitations regarding this library are associated with the toxicity of some antigens and some ethical issues, which consequently impair the feasibility to active immunization of humans or other animals for obtention of antibody repertoires [41, 44].

The naïve APLD involves the generation of libraries that allows the discovery of MAbs against all types of antigen. These libraries are produced through the repertoires of healthy donors, and antibody genes contained have much more diversity than immune libraries. The main advantage of using naïve libraries is the possibility to isolate MAbs against non-immunogenic and toxic antigens. However, the major drawback is that the selected MAbs often have low affinities compared with antibodies from immune libraries [33]. The semisynthetic APLD is based on the display of artificially made diversity in V-gene segments, usually by in vitro randomization of CDRs from a limited number of naïve variable regions, reconstructing the V-gene repertoires [41]. One of the characteristics of these libraries is the absence of natural biases and redundancies usually found in a naïve library. Unlike semisynthetic APDLs, the

fully synthetic library is constructed through the incorporation of nucleotide randomization based on in silico design and de novo synthesis. This refined synthesis appears to increase the functional size of library and consequently the isolation of MAbs with a great range of affinity. However, they still need to be optimized regarding their binding sites, affinity, valency, and other characteristics [41, 44].

Except in the case of fully synthetic libraries, generally, the construction of an antibody library is based on the amplification of the repertoire of the variable chain genes of one or more individuals using primers that cover all families of this gene. Subsequently, a random combination of the VL and VH chain is generated. In the case of the production of Fab libraries, a step is taken to join each variable chain fragment with its respective constant region. The PCR products of these amplifications, representing the antibody repertoire, are ligated into a phagemid vector and transformed into *E. coli*. However, phage vectors generally have only the origin of replication of the phage; they do not contain all the genes necessary for replication and assembly of these phages. Thus, screening libraries using this phagemid requires a helper phage to provide replication and assembly proteins. The addition of the phage to the bacterial cells transformed with the phagemid will result in the production of a mixture of phages that will present predominantly the phagemid vector [45].

Once assembled, each phage exposes a fragment of functional antibody fused to one of the phage surface proteins [46, 47]. A determining factor for the quality of a library and consequently the success of a biopanning by Phage Display is its initial diversity, given by the number of different antibodies in the library. The greater the initial diversity of clones within the library, the greater the likelihood of containing sequences that will bind to a given target with greater affinity [45]. The capacity to produce very large libraries (1012 different clones) has turned the Phage Display into a fast and reliable high-throughput screening methodology [43].

Most biopanning methods are based on four main steps, preparing a library; incubation of that library with a given antigen; removal of nonspecific or low-affinity phages; and recovery of binding targets, which will be amplified after infection in *E. coli* and used in the next biopanning cycle (**Figure 2**). The Phage Display biopanning process is characterized by an increase in the number of clones with affinity for the target molecule through successive selection cycles, with a consequent reduction in the diversity of clones and in the presence of clones with low affinity [48]. Thus, the biopanning results in the sequential enrichment of phages that have a specific binding to the antigen.

These biopanning steps are usually repeated three to five times, until a high specificity/affinity ligand is identified [34, 45, 46]. During biopanning, phage binding to the antigen is retained on the plate, and, after a series of washes, these phages are eluted and amplified. Subsequently, the phages are again incubated with the antigen in the next cycle. Phages with low affinity for antigens may stick to the plate, not interacting with the particle, or remain suspended in the solution. After the wash step, many of these nonspecific phages are removed. Generally, this step involves the application of a greater wash stringency in each subsequent round, which can be performed by increasing the number of washes or increasing the concentration of the nonionic detergent buffer used.

3.2 Post-selection step

During the biopanning stage, it is possible to monitor the enrichment of antibodies by measuring the phage titers that enter the selection and the phage titers that are eluted, assessing the enrichment ratio at each selection cycle. The enrichment follow-up provides the assurance that the selection was carried out

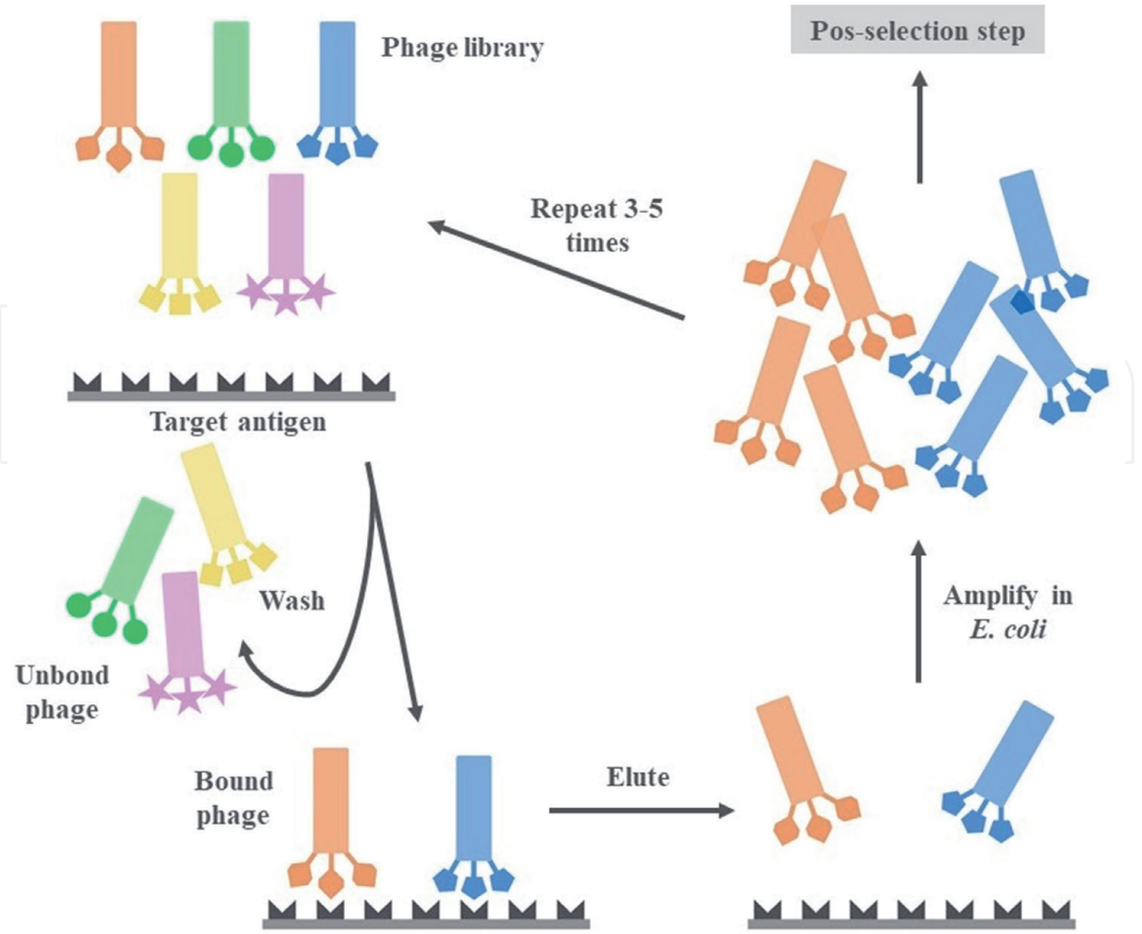

Figure 2.
Biopanning steps. Representation of a biopanning process characterized by a step of Phage Display library incubation with the target antigen, removal of unbound phages, elution of bound phages, and phage amplification in E. coli*, followed by another cycle. After 3–5 cycles post-selection step is carried out.*

efficiently and is followed by the analysis of the selection progress and the identification of the antibodies that have greater affinity to the target antigen [49]. Over the decades, different ways of analyzing selection have been reported, depending on the selection system employed, the antibody library used, and the antigen of interest. However, two methods stand out for the quality of the results they offer, a monoclonal analysis of a sample of the selected antibodies and a polyclonal analysis of the sequences of the entire antibody population.

From the population of selected phages or using cultures of the selected soluble antibodies, the specific binding of individual clones to the immobilized antigen is assessed in an ELISA assay. In this monoclonal ELISA, the binding of 30–100, or more, randomly chosen clones is compared with each other and with the negative control. Phages with the highest absorbance values are considered the ones that have displayed functionally antibodies of greater affinity. Positive clones for the binding analysis are subjected to a Sanger sequencing reaction to determine the sequences of the antibodies [50–52]. In this type of Phage Display analysis, the phenotypes (activity) of the antibodies are investigated first and then their genotype is determined. The disadvantage of this type of analysis is that it does not allow exploring the antibody population in depth and may not include all antibodies of greater affinity. In addition, it does not allow the study of the magnitude of selection and enrichment [53].

A high-throughput sequencing provides a tool for rapid analysis of the selection and direct identification of the most enriched antibodies, with greater affinity,

without requiring a step of their expression. In addition to the speed of analysis, it is possible to investigate the original diversity of the library; identify all antibodies that enriched, the most enriched, and the rare in the population; and determine the frequency of increase throughout the selection. This is possible because sequencing technologies, called next-generation sequencing (NGS), are used, and they allow sequencing a large number of sequences, in the order of millions, in the same sequencing reaction [54]. The interpretation of NGS results from antibody libraries requires the use of a bioinformatics tool specialized in calculating the enrichment of variable domains in a selection of Phage Display. Different tools for this purpose are described in the literature, such as the recent ATTILA pipeline [55].

Despite providing a profound assessment of all antibodies in all cycles, two major problems arise in the analysis of biopanning by sequencing. The first corresponds to the noise in the identified final sequences that results from the sequencing process or the gene amplification reaction. However, more accurate pipelines for isolating DNA libraries for sequencing and more powerful bioinformatics analysis programs have been produced to overcome the artifacts introduced by PCR and sequencing errors. The second is the limitation of the high-performance sequencing methodology that, although it allows the sequencing of millions of sequences, can only properly read up to 400 base pairs. Therefore, this analysis requires that the variable domains of the heavy and light chain of antibodies, whose size ranges from 300 to 400 base pairs, be amplified and sequenced separately, resulting in the loss of the VH and VL pairs of the most enriched antibodies. Recently, studies have been carried out to provide a method of sequencing without losing the VH and VL pairs of antibodies. In the analysis of the selection process by a high-throughput sequencing, the antibody's genotype is first determined and then their phenotype is characterized [53, 56, 57].

4. Application of Phage Display in the context of DENV and other flavivirus infections

The use of MAbs against an infection pathogen is an area of great interest for research. In **Table 2** it is demonstrated MAbs developed for infection disease who have been approved by the FDA. As can be seen, few MAbs are approved for use in infectious diseases, although there is still a strong demand for development in this field. Some challenges involving MAb production against pathogens are their economic viability due to their high cost and if target an episodic disease, there is no supporting for continued production. Moreover, there is a concern about the selection of neutralization-escape mutants [58]. However, they may be notably effective for certain emerging infectious diseases, in which the process of vaccine development could be lengthened and difficult. Thus, MAbs should have more effectiveness for the first response against these diseases [57].

Small molecules are most antibiotic antivirals. However, Phage Display-derived MAbs have an overall success rate of 35% of passage from clinical phase I to launch, compared to an average of 12% for a small-molecule drug candidate [59]. In this regard, Phage Display-derived MAb is considered an important alternative approach to infectious disease treatment compared to classical small-molecule discovery. Raxibacumab is an example of a fast-track designation from the FDA, providing the expedition of the drug to use against *B. anthracis* infection. This bacterium secretes proteins, the lethal factor and the edema factor, that inhibit normal immune system functioning that ultimately cause cell death. The entry of these factors is mediated by the protective antigen (PA), also secreted by the bacteria. Raxibacumab is directed to *B. anthracis* PA and thus prevents the cellular

Antibody	Target	Format	Indication	Company	Year	Method
Pending Raxibacumab	PA	Human	Anthrax infection (*Bacillus anthracis*)	GSK	2012	Phage Display
Zinplava Bezlotoxumab	Toxin B	Human	*Clostridium difficile* infection	Merck/ Dohme	2016	Transgenic mice
Trogarzo Ibalizumab	CD4	Humanized	HIV infection	TaiMed	2018	Hybridoma
Synagis Palivizumab	RSV F	Humanized	Respiratory syncytial virus (RSV) prophylaxis	MedImmune	1998	Hybridoma

PA, protective antigen; CD4, cluster of differentiation 4; RSV F, respiratory syncytial virus fusion

Table 2.
Monoclonal antibodies for infectious diseases approved by the FDA.

uptake of the lethal factor and edema factor. The MAb was developed by the Phage Display, using a library licensed by Human Genome Sciences (HGS), which now is GlaxoSmithKline (GSK), from Cambridge Antibody Technology. Recombinant PA was used in the biopanning process to select candidates, which were then screened in assays for PA neutralization [60, 61].

The Phage Display technology provides a rapid methodology for building a high-affinity antibody library from immune repertoires. These antibodies can be used to generate diagnostic bases or be tested for therapeutic capability. For example, from the repertoire of B cells of patients who recovered from *influenza* virus infections or who received vaccination, it was possible to isolate, by Phage Display, several antibodies with the neutralization property of different *influenza* virus subtypes. Another example of antibody-based immunotherapy developed by Phage Display involves the identification of antibodies specific to different types of coronavirus. These studies are an example of how Phage Display enables the selection of antibodies by an in vitro process, especially for new or mutated pathogens in an outbreak of emergent infectious diseases, as it uses only pathogen-specific antigens [39, 62, 63].

Particularly, MAbs play an important role in antiviral immunity preventing viral replication and disease progress. Antibodies can interfere with virus infection by various mechanisms. The primary mechanism is by targeting the virus surface proteins; antibodies can inhibit virus attachment to cell surface receptors. Another main mechanism is targeting non-receptor-binding regions, such as in endosomal membrane fusion step where neutralization can occur by interfering virus conformational changes. In general, flavivirus particles tend to display on their surface continuum epitopes that induce potently neutralizing antibodies, blocking viral entry into cells [58].

From the understanding of the structure of each flavivirus, it was possible to determine the antibody targets most conducive to the diagnosis and protection of the disease [64]. It is important to note that flaviviruses are not static particles and viral proteins are in a constant dynamic movement, a process known as breathing, in order to transiently reveal new epitopes, and this characteristic influences the detection and neutralization capabilities of antibodies [10].

So far, no MAbs against flavivirus have reached the clinical stages, except for WNV. However, several studies have demonstrated potentially neutralizing MAbs that could be therapeutically used against these infections [65, 66]. Different antibodies have been generated exploring the characteristics of viral epitopes. The E glycoprotein is the main target of neutralizing antibodies, especially the E DIII has

been described to be the most efficient to block adsorption of DENV in vitro [58]. In the field of DENV diagnosis, MAbs have been especially applied to distinguish DENV serotypes [67, 68]. For this purpose E and soluble NS1 proteins are the main targets of these MAbs using different assay formats, such as ELISA and rapid test based on immunochromatography [69].

The pre-existing cross-reactive antibodies can be boosted in a secondary infection with antigenically related molecules; consequently antibody to fusion loop tends to have dominance upon sequential infections with DENV or other flaviviruses. Antibodies to E-dimer epitope (EDE) are divided into two subclasses, EDE1 and EDE2, based on the recognition of the conserved glycan Asn-153 of DENV [58]. EDE1 has already been shown to potently neutralize ZIKV infection; this class of antibody does not require glycosylation for binding [70]. However, EDE2 have a reduced neutralization potential against ZIKV, once these antibodies have a strongly binding dependence on the glycan, which have different positioning between ZIKV and DENV [66].

The generation of monoclonal antibodies by Phage Display can help improve the speed at which new antibodies are produced. The freedom associated with recombinant antibodies also allows them to be customized for various applications, allowing the development of MAbs with binding, functional, and pharmacological characteristics suitable for a therapeutic and diagnostic use [37]. Thus, the use of Phage Display to identify antibodies against DENV, as well as for other flavivirus, can contribute to the knowledge of the specific antigenic properties of the virus, allowing to generate new perspectives for the development of efficient therapies, vaccines, and diagnostic platforms of this virus.

To obtain specific antibodies to the DENV, it is possible to employ different libraries of Phage Display and distinct selection approaches depending on the purpose. Using a llama immune library, a diagnostic methodology was developed based on antibodies capable of binding to the NS1 of the four DENV serotypes, without cross-reacting with NS1 of other flaviviruses. The panning was performed with immobilized antigen, so that in each round, the phage population was incubated with NS1 from one of the serotypes, resulting in phage specific to all forms of NS1. To characterize the diagnostic potential of the antibodies, MAbs were addressed [71]. Lebani et al. [72] isolate four serotype-specific human antibodies through a negative selection strategy. Each MAb was specific for NS1 from a DENV serotype, without cross-linking.

In another approach, Cabezas et al. [73] worked with human naïve library to obtain a panel of antibody fragments with different specificity toward DENV serotypes. The biopanning was made against inactivated DENV-containing supernatants harvested from infected Vero cells for 4 days with each serotype. These supernatants were directly used for Phage Display biopanning. A panel of nine scFvs, where seven were specific for DENV2, DENV3, and DENV4 while the other two were cross-reactive, was obtained. Silva [74] employed a subtractive biopanning, in which a human Fab Phage Display library was first incubated against ZIKV particles, to eliminate the majority of antibodies that binds to this viral particle, and nonbinding phages were then incubated against DENV2 particles, followed by elution of ligand phages. Analysis by NGS of the pool of phages retrieved after four rounds of this biopanning showed that the VH and VL sequences obtained may not have cross-reactivity between DENV2 and ZIKV.

Antibody-based DENV infection therapies developed by Phage Display have also been reported. Saokaew et al. [75] show that a human scFv specific for DIII was able to neutralize DENV2 infection at in vitro assays. The human MAb 5A, originated from a selection of Phage Display, has been shown to be specific to the fusion loop, both in its pre-fusion conformation to the endocytic membrane, before infection, and in its acid-dependent post-fusion conformation, during the final viral infection

process, and proved to be a potent neutralizing antibody of different strains of yellow fever virus in vitro and in vivo. Thus, MAb 5A prevents both virus attachment and fusion. As the fusion loop is a highly conserved antigen, there is a high possibility that 5A neutralizes other flaviviruses [76]. In the same way, Wu et al. [77] identified a panel of human MAbs that target DIII of the ZIKV envelope protein from a large Phage Display naïve antibody library. These germline-like antibodies bound ZIKV DIII specifically with high affinities. These MAbs neutralized the currently circulating ZIKV strains and showed a synergistic effect in neutralizing ZIKV in vitro and in a mouse model of ZIKV infection.

As an example of Phage Display using immune libraries to select high-affinity MAbs, Mwale et al. [78] analyzed the immune response in chicken through the determination of the polyclonal immunoglobulin yolk (IgY) against a truncated Zika virus envelope protein. They induced an immune response in white leghorn laying hens against the ZIKV envelope protein. A high-level titer of anti-ZIKV envelope protein antibodies was detected and after constructed two antibody libraries; they found some scFvs that showed specific binding activities toward the ZIKV envelope protein.

Moreover, Phage Display has been used to find therapeutic antibody fragments against nonstructural proteins. A MAb fragment Fab NS3-specific obtained from a naïve human Fab Phage Display library was shown to inhibit the ATPase and helicase activities of NS3 protein and reduces DENV replication in vitro. The ability to inhibit in vitro DENV replication may be exploited in a therapeutic approach [79]. Using a human scFv Phage Display library, Poungpair et al. [80] obtained two scFv clones that bound specifically to the NS1 of DENV 2, used as antigen in phage biopanning. They observed that cells infected with DENV2 and treated with selected scFvs had significant reduction of the infectious viral particles in supernatant. Besides that, the analysis of mimotope/epitope mapping indicated that the NS1 sites bound by antibody fragments can lead to interference of the virus replication by affecting the virus release.

5. Final considerations

Over the years many discoveries have been made aiming for the control and treatment of emerging infectious diseases, some of those include the development of efficient drugs that could act specifically in the pathogen to eliminate efficiently. In this way, MAbs emerged as the main biological drugs for this purpose. Moreover, MAbs play an important role in the development of serological diagnostic test that could be used for tracking the spread of disease and determining public health prevention measures and clinical care. There are still great questions around the infection mechanisms by flavivirus, especially related to the cross-reactivity between them and the risk of complications. In this way, the use of effective, fast, and robust approaches to facilitate the development of flavivirus MAbs is a determinant factor.

The Phage Display technology presents a great potential to provide optimized strategies, allowing the obtention of high-affinity human antibodies for a specific target. Some of the main advantages that make this technology so promising are the possibility to obtain human MAbs without in vivo immunization; the enormous diversity of variant antibodies displayed within a single library; the ability to tailor MAbs with the desired properties by using different strategies such as depletion, guided selection, and biochemical control; and the possibility to be applied against practically any kind of target antigen [33]. However, some concerns about Phage Display are the dependence of the initial library quality, the difficulties in the post-selection step involving analysis and recombinant antibody production, and the possibility of

obtention of low-affinity antibodies, especially in naïve libraries [35]. Considering the impact of infectious diseases on the health system and economy, mainly DENV and ZIKV, that co-circulate in tropical countries, MAbs obtained by Phage Display may overcome issues related to versatility and high throughputness compared to other approaches, playing a larger role in the actual and future public health response.

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Conflict of interest

The authors declare no conflict of interest.

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