DOI 10.1002/prca.201600002
Proteomics Clin. Appl. 2016, *10*, 922–948

REVIEW

Recombinant antibodies for diagnostics and therapy against pathogens and toxins generated by phage display

Philipp Kuhn[1], Viola Fühner[1], Tobias Unkauf[1], Gustavo Marcal Schmidt Garcia Moreira[1], André Frenzel[1,2], Sebastian Miethe[1] and Michael Hust[1]

[1] Technische Universität Braunschweig, Institut für Biochemie, Biotechnologie und Bioinformatik, Abteilung Biotechnologie, Braunschweig, Germany
[2] YUMAB GmbH, Braunschweig, Germany

Antibodies are valuable molecules for the diagnostic and treatment of diseases caused by pathogens and toxins. Traditionally, these antibodies are generated by hybridoma technology. An alternative to hybridoma technology is the use of antibody phage display to generate recombinant antibodies. This in vitro technology circumvents the limitations of the immune system and allows—in theory—the generation of antibodies against all conceivable molecules. Phage display technology enables obtaining human antibodies from naïve antibody gene libraries when either patients are not available or immunization is not ethically feasible. On the other hand, if patients or immunized/infected animals are available, it is common to construct immune phage display libraries to select in vivo affinity-matured antibodies. Because the phage packaged DNA sequence encoding the antibodies is directly available, the antibodies can be smoothly engineered according to the requirements of the final application. In this review, an overview of phage display derived recombinant antibodies against bacterial, viral, and eukaryotic pathogens as well as toxins for diagnostics and therapy is given.

Received: January 11, 2016
Revised: March 30, 2016
Accepted: May 17, 2016

Keywords:
Antibody phage display / Antibody engineering / Pathogens / Toxins

1 Introduction

Antibodies are essential molecules as tools for basic research [1], diagnostics [2], and therapy [3]. In the past—and still today—polyclonal antibodies were produced as serum in animals like horses [4]. A milestone in antibody generation was the development of hybridoma technology that allows the production of monoclonal antibodies [5]. But the hybridoma technology has drawbacks like limited number of candidates, possible instability of the aneuploid cell lines [6], inability to provide antibodies against highly conserved antigens and most of all its limited application to generate human antibodies [7]. The hybridoma technology essentially gives murine antibodies that can be used for diagnostic or research purposes. However, their therapeutic applications are limited because repeated administration of murine antibodies can cause human anti-mouse antibody reaction, reducing antibody half-life and leading to severe side effects such as anaphylactic shock [8]. A strategy to circumvent these problems is antibody humanization or the use of transgenic animals in which the original antibody gene repertoire is replaced with a human gene repertoire [9–12]. Another strategy is the human hybridoma technology resulting in human antibodies [13, 14], although this technology has the same problems of the murine hybridoma technology regarding the limitation of the immune system.

A technology that overcomes the limitation of the immune system is antibody phage display. Since this technology involves an in vitro selection process, it is completely independent of any immune system. The display method

Correspondence: Dr. Michael Hust, Technische Universität Braunschweig, Institut für Biochemie, Biotechnologie und Bioinformatik, Abteilung Biotechnologie, Spielmannstr. 7, 38106 Braunschweig, Germany
E-mail: m.hust@tu-bs.de

Abbreviations: CDC, Centers for Disease Control and Prevention; **DENV**, dengue virus; **DIVA**, differentiating infected from vaccinated animal; **ET**, edema toxin; **LF**, lethal factor; **MSP-3**, merozite surface protein 3; **PA**, protective antigen; **SEB**, staphylococcal enterotoxin B; **TTX**, tetrodoxin; **VEEV**, Venezuelan equine encephalitis virus; **WEEV**, Western equine encephalitis virus

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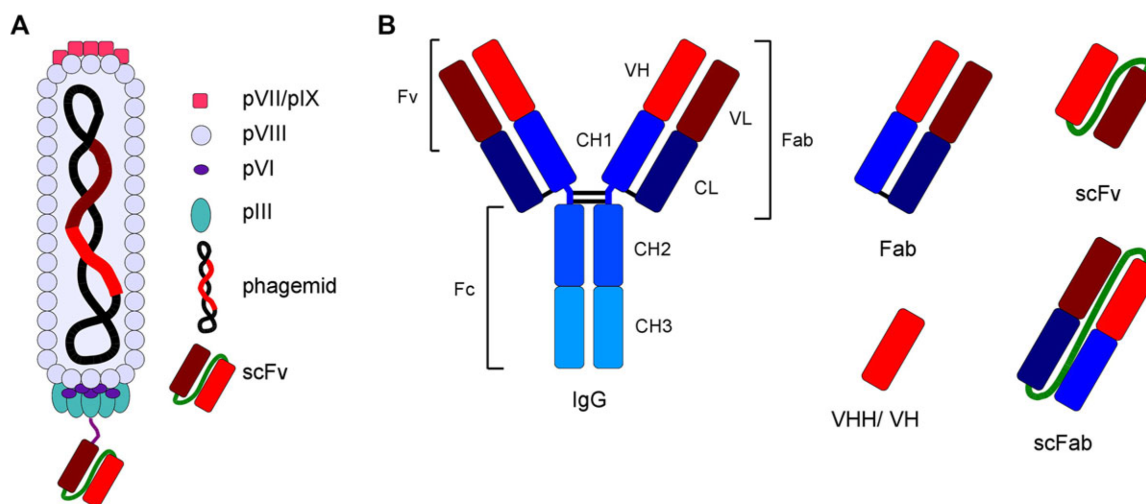

Figure 1. (A) Schema of an antibody (scFv) phage. (B) Different antibody formats used for phage display. Abbreviations: Fab, Fragment antigen binding; Fc, Fragment constant; Fv, Fragment variable; pIII, pVI, pVII, pVIII, pVIX, phage protein III, VI, VII, VIII, VIX; scFab, single chain Fab; scFv, single chain fragment variable; VH, variable domain heavy chain; VHH, variable domain of the camel heavy chain.

most commonly used today is based on the work of Georg P. Smith on filamentous phage, which infect *E. coli* [15]. The selection process is called "panning," referring to the gold digger's tool [16].

M13 phage display technology was further developed 1990/91 for antibodies in three places in parallel: Heidelberg (Germany), Cambridge (UK), and La Jolla (USA) [17–20]. Two different genetic systems have been developed for the expression of the antibody::pIII (phage protein III) fusion proteins for phage display. First, the antibody genes can be directly inserted into the phage genome fused upstream of the wild-type pIII gene [20]. Second, most successful systems, uncouple antibody expression from phage propagation by providing the genes encoding the antibody::pIII fusion proteins on a separate plasmid (called "phagemid") that contains a phage morphogenetic signal for packaging the vector into the phage particles [18]. No matter the used phage display system, antibody fragments are displayed on the surface of M13 phage and the corresponding antibody gene is packaged in the phage particle. Other phage display technologies using phage lambda [21] or T7 [22] are less suitable for the display of antibody fragments. In addition, these phages are lytic phage that aggravates the practical work. The most common antibody formats used for antibody phage display are the single chain fragment variable (scFv) [23–25] or fragment antigen binding (Fabs) [26, 27]. Other antibody formats used for phage display are single chain Fabs (scFab), human VH domains (dAbs), the variable domains of camel heavy chains (VHHs) and immunoglobulins of sharks (IgNARs) [28–33]. Figure 1 shows an antibody phage and different antibody fragments. For veterinary research, chicken libraries are often used [34, 35]. In contrast to what occurs in humans, the diversity of chicken antibody genes is the result of gene conversion, and the N- and C-terminal parts of chicken's VH and VL are always identical, facilitating antibody gene amplification, and library cloning [36, 37]. Also rabbit phage display was used to generate antibodies against pathogens [38].

In the selection process (panning), the antigen is immobilized to a solid surface, like column matrixes [18], magnetic beads [39], or plastic surfaces with high protein-binding capacity such as polystyrene tubes or respectively microtitre wells, which is the most common method [40]. An other option is the panning in solution using biotinylated antigens followed by a "pull-down" with streptavidin beads [41]. The vast excess of nonbinding antibody phage will be removed by stringent washing. Subsequently, the bound antibody phage will be eluted, e.g. by pH shift or trypsin, and reamplified by infection of *E. coli*. After infection of the phagemid bearing *E. coli* with a helperphage, new antibody phage will be produced. The selection cycle will be repeated and the number of antigen-specific antibody phage clones should increase with every panning round. Usually 2–3 panning rounds are performed. Finally, monoclonal antibody phage or monoclonal soluble antibodies can be identified by, e.g. ELISA [42], immunoblot [40], or flow cytometry [43]. The antibody fragment genes can be subcloned into any other antibody format, e.g. scFv-Fc or IgG [23, 27, 42, 44, 45]. A schema of the selection process is given in Fig. 2.

Regarding the source of genes, antibodies can be selected from two types of libraries: immune libraries and universal libraries. Immune libraries are constructed from immunized/infected donors and typically used in medical research to obtain an antibody against a particular target antigen, e.g. an infectious pathogen like Ebola virus [46]. An advantage of this kind of library is that the V-genes contain hypermutations and are affinity matured, although its development can be restricted to ethical constraints.

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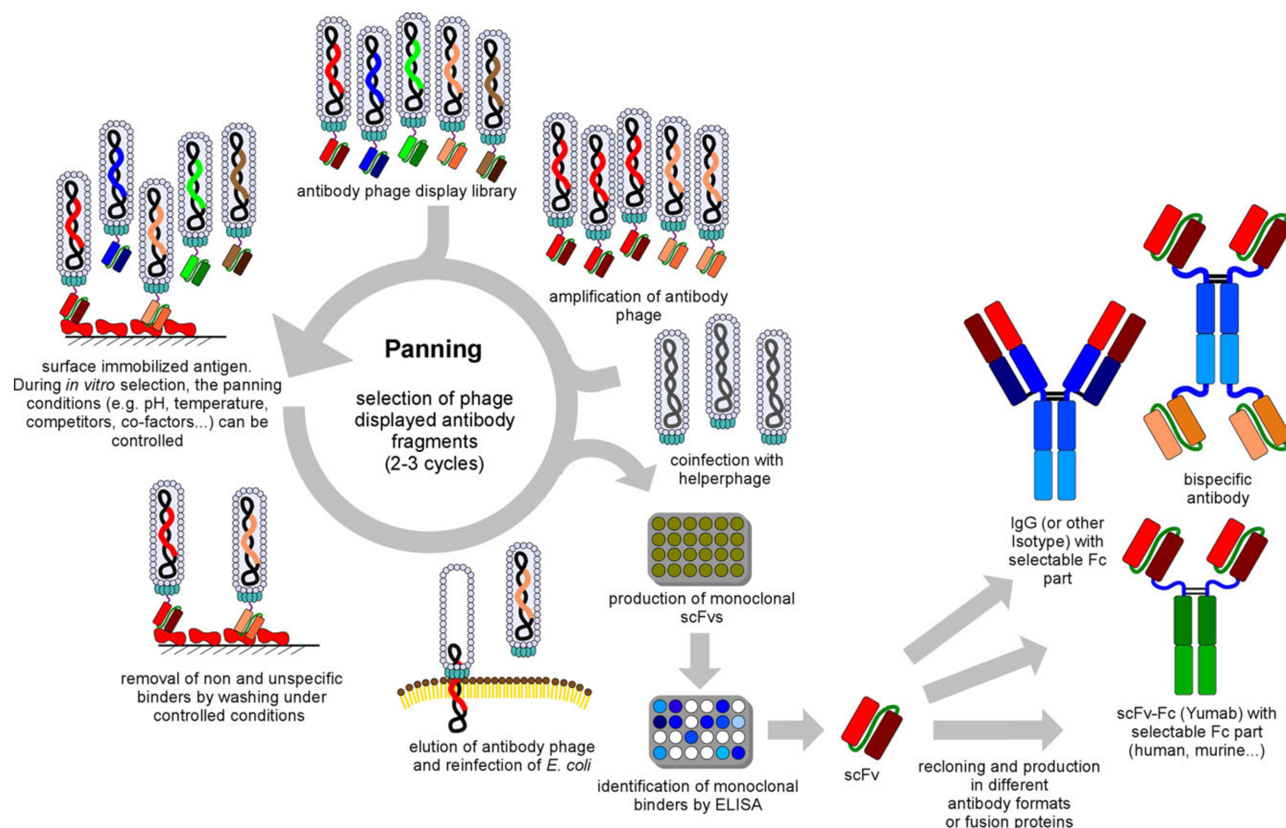

Figure 2. Schema of antibody selection using phage display.

The alternative are universal or “single pot” libraries, which includes naïve, semisynthetic and synthetic libraries that are designed to isolate antibody fragments binding to every possible antigen, at least in theory [44, 47]. Naïve libraries are constructed from rearranged V genes from B cells (IgM) of nonimmunized donors. Examples for this library type are the naïve human Fab library constructed by de Haard and colleagues [26] and the HAL scFv libraries [23, 48]. Semisynthetic libraries are constructed from unrearranged V genes from pre-B cells (germline cells) [49] or from one antibody framework [50] in which one or several CDRs, but always the CDR H3, are randomized. Often used semisynthetic libraries are the Tomlinson I and J libraries using one defined framework VH3-23 and Kappa IKV1-39 with randomized CDR2 and CDR3 [51].

A combination of naïve and synthetic repertoire was used for the FAB310 antibody gene library. In this library, light chains from autoimmune patients were combined with a Fd fragment (VH+CH1) containing synthetic CDR1 and CDR2 in the human VH3-23 framework and naïve CDR3 regions, originated from autoimmune patients [27]. Fully synthetic libraries are made of human frameworks with randomized CDR cassettes [52–54]. The theoretical size of these universal libraries is usually higher than 10^{10} independent clones [24, 48, 54–56].

To date, 53 antibodies and antibody conjugates were approved by EMA and/or FDA (status January 2016) (http://www.imgt.org/mAb-DB/query.action, Development status: Phase M in search field) and about 350 antibodies were under development in 2013 [57]. Most approved therapeutic antibodies are for cancer and autoimmune diseases and the annual sales of therapeutic antibodies exceeded 50 billion US$ in 2013 [58]. The mechanisms of therapeutic antibodies are manifold and include neutralization of substances, e.g. toxins [59] or cytokines like tumor necrosis factor alpha [60], blocking of receptors like epidermal growth factor receptor [61], binding to cells and modulating the host immune system [62], or combinations of these effects [63]. Currently, two recombinant antibodies are approved for the treatment of pathogens or toxins. Raxibacumab is a human antibody for anthrax treatment derived from a phage display library from Cambridge Antibody Technology (now Medimmune, part of AstraZeneca) in cooperation with Human Genome Science (now GlaxoSmithKline) [64]. The antibody palivizumab for the treatment of Respiratory syncytial virus bronchiolitis is a classical humanized antibody [65]. A further antibody, but also not derived from phage display, the *Clostridium difficile* antibody bezlotoxumab is in clinical phase 3 [66].

An overview of recombinant antibodies derived from phage display against bacterial and viral pathogens,

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eukaryotic pathogens (parasites, fungi), and toxins as well as detailed examples for diagnostic and therapy are given in the next sections.

2 Recombinant antibodies against bacteria

The most therapeutic antibodies against bacterial targets are generated against toxins. These antibodies are described in the section "recombinant antibodies against toxins." The majority of antibodies against bacteria are developed in order to facilitate diagnostics in patients [67, 68] and environmental samples [69, 70]. In general, cultural and microbial detection of bacteria is regarded as standard in diagnostics for many pathogens, e.g. *Mycobacterium tuberculosis* [71] or *Salmonella* Typhimurium [72]. Since these methods are often time-consuming and require experienced lab personal, high throughput analysis is often limited. Real-time PCR measurements have been developed for the diagnostics of many bacteria [73], offering high sensitivity and specificity of detection. But sample treatment is still needed in many cases, including expensive and complex laboratory devices [74]. An other approach is MS for diagnostic, but this technology needs expensive devices [75]. Antibody-based diagnostic like ELISA would be more simple and easy to use, also in developing countries.

In order to generate antibodies with the desired-binding properties, phage-display has been used to select antibodies on proteins or polysaccharides of *Chlamydophila psittaci* [76], *Chlamydia trachomatis* [77], *Haemophilus influenzae* [78], *Listeria monocytogenes* [79], *Mycobaterium bovis* [35], *Mycobacterium tuberculosis* [68, 80, 81], *Porphyromonas gingivalis* [67], *Ralstonia solanacearum* [69], *Salmonella* Typhimurium [2, 82], and *Yersinia pestis* [83]. But even selections on cell lysate or whole cells were performed on *Mycobacterium avium* [84], *Bacillus anthracis* [70, 85], *Moraxella catarrhalis* [86], *Lawsonia intracellularis* [87], *Lactobacillus acidophilus* [88], *Helicobacter pylori* [89], *Brucella melitensis* [90], and *Bordetella pertussis* [91]. In the following paragraphs, we give detailed examples for antibody generation using phage display and antibody engineering against different bacterial pathogens.

Tuberculosis (TB) is a bacterial infection caused by *Mycobacterium tuberculosis* (Mtb), a pathogen with particular cell wall and membrane characteristics that is able to infect and multiply within alveolar macrophages leading to cough, weakness, and fever [92]. In 2013, 9 million people were infected and 1.5 million died worldwide [93]. In order to improve treatment and control disease spread, TB must be diagnosed as early as possible. One prominent target for this purpose is the bacterial Antigen 85 (Ag85) that is the most abundant protein secreted by Mtb [94]. It is a complex of three highly homolog proteins (Ag85A, Ag85B, and Ag85C) with a molecular size of 30–32 kDa each [95]. In a novel approach by Ferrara et al. [68] monoclonal antibodies were selected against the Ag85 complex by combining phage- and yeast-display. First, a naïve scFv-library was preselected against Ag85 by phage display, reducing the diversity from $\sim 10^{11}$ unique scFv clones to $\sim 10^{5}$ colony forming units. Second, the enriched sublibrary was cloned into a yeast-display system. Each clone of the selection output was screened individually for antigen binding by FACS. Those with the highest antigen-binding signals were sorted and further analyzed. In total 192 clones were sequenced revealing 111 genetically unique scFv clones. Further screening assays identified seven antibody pairs, which can detect Ag85 down to a concentration of 6.1 nM in the absence of serum and 22.7 nM in its presence. Interestingly, none of the antibodies were absolutely specific for one of the Ag85 subunits of the complex. All three subunits A, B, and C were bound although not equally. In contrast, Fuchs et al. selected five human antibodies against the recombinant Ag85B protein from the naïve HAL7/8 libraries [81]. Three of them bound specifically to the 85B protein in ELISA (LOD: 5 ng/mL). Even sandwich detection of recombinant 85B in ELISA (LOD: 10 ng/mL) and integration into lateral flow immuno assay (LOD: <5 ng/mL) was shown. But antigen detection in Mtb cell extracts or culture filtrates was restricted to direct ELISA and immunoblot assay. The authors of both papers argued that the selected antibodies must be affinity matured in order to reach required detection limits. Successful implementation of this concept was demonstrated by Sixholo et al. [80]. Chicken antibodies were selected from the semisynthetic Nkuku library against a 16 kDa recombinant antigen of *M. tuberculosis*, also known as heat shock protein X (HspX). After four rounds of panning, three scFvs were identified in ELISA. The clone with the lowest ELISA signal was selected for in vitro engineering using two different approaches. First, a mutant library was generated from the parental scFv by error-prone PCR with a diversity of $\sim 3 \times 10^{7}$ unique scFv. This library was selected again on the HspX antigen under more stringent conditions, leading to identification of three mutant scFvs. These contained one or three individual amino acid exchanges, occuring both in CDR and framework regions. All mutants showed increased ELISA-binding signals, reaching a signal $\sim$11 times higher when compared to the parental scFv. Furthermore, the mutants showed improved association and dissociation kinetics in ELISA and SPR analysis. In the other approach, the length of the scFv linker was reduced from 15 amino acids to a single glycine residue, forcing tetramerization of antibodies. Due to cooperative binding, the apparent functional affinity was improved. In ELISA, tetrameric scFv generated a $\sim$14 times higher ELISA signal, and SPR proved increased association and decreased dissociation rates.

Porphyromonas gingivalis is one of the major pathogens involved in periodontitis [96]. Periodontitis is an inflammatory disease, which causes the loosening and loss of teeth. Secreted cysteine proteases like RgpB contribute to disease pathology and represent potential biomarkers for disease detection and progression [97]. Skottrup et al. [67] generated a naïve antibody library from camels with a diversity of $\sim 5 \times 10^{7}$ clones. A special feature is that the antibodies consist of a

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single monomeric VHH domain, also called nanobodies. Advantages of this format are the small size (~15 kDa), the ease production in *E. coli*, and the convex paratope that is formed which enables the targeting of cryptic epitopes. The library was selected on immobilized RgpB. One clone was isolated that binds specifically to cell surface displayed and soluble RgpB with an affinity of 362 pM. A detection limit of $\sim 8 \times 10^6$ cells/mL of saliva was reached when tested by subtractive inhibition ELISA. But catalytic activity of RgpB was not inhibited by antibody binding.

Salmonella Typhimurium, is one of the most important pathogens of foodborne gastrointestinal infections [98]. Meyer et al. [82] first identified novel immunogenic proteins of *S*. Typhimurium from a genomic library using oligopeptide phage display. In a second step human antibodies were selected against these targets from the naïve HAL gene libraries. For DIVA (differentiating infected from vaccinated animals) vaccine development, *S*. Typhimurium strains lacking a marker protein, e.g. OmpD (outer membrane protein D) were developed (DIVA vaccines) [99]. To allow the discrimination between vaccinated animals and infected animals a diagnostic of this specific marker is necessary. Here, Meyer et al. [2] generated scFvs against OmpD for diagnostics purposes. Only some of the generated antibodies were suitable for a competitive ELISA using swine serum showing also the difficulties when developing diagnostic assays that are often hampered by the complexity of serum samples.

An overview of recombinant antibodies generated by phage display against bacterial pathogens is given in Table 1.

3 Recombinant antibodies against viruses

Up to now, a large panel of antibodies against various viruses has been generated from either naïve or immune libraries using phage display technology. Panning against peptides, recombinant viral proteins, or complete virus particles has led to the identification of antibodies directed against human pathogenic viruses such as Sin nombre virus [100], Dengue virus [101, 102], Influenza virus [103, 104], VEEV [105], Norovirus [106], SARS coronavirus [107], or Hepatitis C [108] from naïve antibody gene libraries. Other antibodies were selected from immune antibody gene libraries targeting, e.g. Western equine encephalitis virus (WEEV) [109], HIV [110, 111], SARS [112], Yellow fever virus [113], or Influenza virus [114, 115]. Semisynthetic libraries were also used to generate antibodies specific for Influenza virus [116]. Beside human and animal viruses, antibodies were also generated against plant viruses [40, 117, 118].

Libraries originated from different species have been successfully employed to isolate virus-specific antibodies in the past such as those from macaque [119], mouse [120], chimpanzee [121], llama [122], chicken [123], and human origin [124].

Most of the virus-specific antibodies have been isolated from libraries in scFv format [125, 126], although Fab [127, 128] and VHH libraries [122] were also successfully used. An interesting approach was used by Xiao et al. using the antibody CH2 domain as scaffold to generate binders against gp120 of HIV [129].

Different antibody characteristics have an influence on virus binding and neutralization. Neutralizing antibodies prevent cell binding of the virus. The anti-gp41 antibody HK20 has a higher neutralizaton rate as scFv or Fab compared to IgG showing, that the epitope is less accessable for the IgG format [130]. Another interesting example is the anti-gp41 VHH 2H10. Here, a tryptophan in the CDR3 is not relevant for epitope binding, but essential for virus neutralization [131].

In the following paragraphs, we give detailed examples for antibody generation using phage display and antibody engineering against different virus groups.

Vaccinia virus is the prototype virus in the genus of O*rthopoxvirus*. It is a relatively large DNA virus with a genome of about 200 kbp [132]. The genus O*rthopoxvirus* includes various species such as monkeypox virus, cowpox virus, and especially variola virus that is the causative agent of smallpox in humans. Naturally occurring smallpox has been eradicated in 1977 because of a massive WHO vaccination program that began in 1967. However, no vaccination of the civilian population is conducted nowadays. The potential threat of intentional release has renewed the search for safe and effective smallpox vaccines as case fatality rates of 30% or more among unvaccinated subjects are reported [133]. Because orthopoxviruses are highly related, it is assumed, that immunity against one poxvirus goes along with immunity against most members of the entire virus family [132, 134]. Using an immune scFv phage display library constructed from vaccinia virus immunized patients, a panel of human vaccinia-specific antibodies was selected. Plaque-reduction neutralization tests revealed that seven of these antibodies neutralized vaccinia as well as cowpox virus in vitro. Five of those antibodies additionally neutralized monkeypox virus [124]. Other antibodies were generated from a Fab immune library derived from vaccinia virus immunized chimpanzee. Converted into a chimeric chimpanzee/human IgG format, two antibodies displayed high affinities to vaccinia protein B5 (K_d of 0.2 and 0.7 nM). Antibody 8AH8AL was neutralizing in vitro for vaccinia and smallpox virus and proofed to be protective in mice challenged with vaccinia virus even when administered 2 days after challenge. In this model 8AH8AL proved to provide significantly greater protection than that of the previously isolated rat anti-B5 antibody 19C2 [135]. Vaccinia had to be used as model, because the final confirmation of protection against smallpox is not possible.

Ebola virus and Marburg virus, two filoviruses, cause severe hemorrhagic fever and possess high mortality of up to 90% in humans. In addition to public health concerns associated with natural outbreaks, Ebola virus might be a potential agent of biological warfare and bio-terrorism [136]. Human

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Table 1. Recombinant antibodies derived by phage display against pathogenic bacteria

Bacteria	Target	Library type	Antibody format	Antibody origin	Application	Reference
Bacillus anthracis	S-layer protein EA1	Immune	VHH	Llama	ELISA, immunoblot	[85]
Bacillus anthracis	Live bacteria	Immune	scFv	Mice	ELISA, immunoblot, IF	[70]
Bordetella pertussis	Filamentous hemagglutinin, pertactin	Immune	scFv	Mice	ELISA, in vitro inhibition, in vivo studies	[91]
Brucella melitensis	Radiated bacteria	Immune	scFv	Mice	ELISA	[90]
Chlamydophila psittaci	2.4[2.8]2.4-linked Kdo tetrasaccharide	Immune	scFv	Mice	ELISA, IF	[76]
Chlamydia trachomatis	Elementary bodies	Naïve	scFv	Human	ELISA, immunoblot, IF	[77, 217]
Clostridium difficile	Different surface proteins (including FliC and FliD)	Semisynthetic	scFv	Human	ELISA, immunoblot, in vitro motility assay	[218]
Haemophilus influenzae	Capsular polysaccharide	Immune	Fab	Human	ELISA	[78]
Helicobacter pylori	Bacterial lysate	Immune	scFv	Human	ELISA	[89]
Lactobacillus acidophilus	S-layer protein	Naïve	scFv	Human	FACS, immunoblot	[88]
Lawsonia intracellularis	Live bacteria	Semisynthetic	scFv	Human	ELISA, IF	[87]
Listeria monocytogenes	Internalin B	Immune/naïve	VHH	Alpaca, llama, camel	ELISA	[79]
Moraxella catarrhalis	HMW-OMP	Semisynthetic	scFv	Human	ELISA, FACS, immunoblot, in vitro inhibition	[86]
Mycobacterium avium	Cell lysate	Immune	scFv	Sheep	ELISA, immunoblot, FACS, IF	[219]
Mycobaterium bovis	HSP65	Semisynthetic	scFv, scFv-IgY-Fc	Chicken	ELISA, immunoblot	[35]
Mycobacterium tuberculosis	Antigen 16 kDa (HspX)	Semisynthetic	scFv	Chicken	ELISA	[80]
Mycobacterium tuberculosis	Antigen 85B	Naïve	scFv, scFv-Fc	Human	ELISA, immunoblot, lateral flow strip assay	[81]
Mycobacterium tuberculosis	Antigen 85	Naïve	scFv, scFv-Fc	Human	ELISA	[68]
Porphyromonas gingivalis	RgpB	Naïve	VHH	Camel	ELISA	[67]
Ralstonia solanacearum	LPS	Naïve	scFv	Human	ELISA, IF, immunoblot	[69]
Salmonella Typhimurium	OmpD	Naïve	scFv	Human	ELISA	[2]
Salmonella Typhimurium	5 different immunogenic proteins	Naïve	scFv	Human	ELISA	[82]
Yersinia pestis	F1	Naïve	scFv	Human	ELISA	[83]

ELISA, enzyme-linked immunosorbent assays; IHC, immuno histo chemistry; FACS, fluorescence-activated cell sorting.

antibodies directed against Ebola virus were selected from a library originated from patients that recovered from infection in the 1995 Ebola virus outbreak in Kikwit, Democratic Republic of Congo [137]. Several antibodies against various viral proteins such as nucleoprotein, envelope glycoprotein, and secreted envelope glycoprotein have been isolated in this study. One antibody (KZ52), specific for envelope glycoprotein, was able to neutralize in vitro as Fab (50% neutralization at 0.4 μg/mL) and as full IgG (90% neutralization at 2.6 μg/mL) [46]. Follow-up studies were showing effective protection in vivo in a Guinea pig Ebola challenge model when the antibody was administered up to one hour

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postviral challenge [138]. Interestingly, KZ52 was not protective in macaques challenged with Ebola even if the antibody was given as a two-dose treatment with the first dose one day prior viral challenge and the second dose 4 days postchallenge [139]. A murine scFv and two shark IgNAR V immune libraries were generated against inactivated Zaire Ebola virus to yield various antibodies specific for the viral matrix protein VP40 and the viral nucleoprotein [136]. Interestingly, this work represents the first example of a successful targeted IgNAR V isolation from a shark immune response library.

Dengue virus (DENV), a member of the *Flaviviridae* family, is responsible for at least 100 million symptomatic infections each year and became a major health and economic burden in over 50 countries worldwide [140–142]. It is a positive strand RNA virus with a $\sim$11 kb genome, that compromise a single open reading frame. The four circulating serotypes of dengue virus show approximately 70% sequence homology [128, 141]. Fab monoclonal antibodies to dengue type 4 virus were isolated from a chimpanzee immune library. Two Fab, namely 5H2 and 5D9 neutralized DENV-4 efficiently with a titer of 0.24–0.58 μg/mL by plague reduction neutralization test [143]. Another study selected human scFv antibodies specific to dengue virus envelope protein by panning against recombinant full-length envelope protein and its domain III [102]. Because DENV envelope protein is an essential molecule for virion assembly and virus entry, scFvs selected in this study were shown to exhibit inhibitory effects on DENV infection in vitro [102]. Dengue nonstructural protein 5 (NS5) is essential for viral replication and host immune response modulation, making it an excellent target for dengue-inhibiting antibodies. A naïve human Fab-phage library was screened for NS5-specific antibody fragments using various NS5 variants from Dengue Virus serotypes 1–4 as antigens for panning and characterization [128]. Using NS5 from alternating dengue serotypes for each round of panning, this strategy resulted in the identification of two clones that are cross-reactive against all four dengue serotypes. Another study selected antibodies using phage display by panning with Dengue virus particles directly captured from supernatant of infected Vero cells. Here, highly serotype-specific antibodies were generated. From a total of nine antibodies, seven were shown to be specific to only one serotype. One Dengue-3 selected clone cross-reacted with Dengue 1, whereas another clone showed cross-reactivity with all serotypes despite being selected solely on Dengue 2 particles. Interestingly, all of the obtained antibodies recognized several strains of distinct genotypes within the corresponding serotype [101]. Panning against dengue envelope protein resulted in the identification of an antibody (C9) from a mouse/human chimeric Fab library that cross-reacts with DENV1-3 and neutralizes DENV2 in cell-based assays after conversion into full-length IgG [141]. Besides scFv and Fab, variable domain heavy-chain antibodies (VHH antibodies) were also selected using phage display technology. After four rounds of panning on recombinant DENV 2 NS1 protein, 20 positive clones were selected. Affinity measurements with NS1 revealed a K_D value of 2.79×10^{-8} M for the best VHH antibody P2 [122].

Venezuelan equine encephalitis virus (VEEV), an alphavirus of the Togoviridae family, causes equine epidemics but can also cause encephalitis in humans [144, 145]. Because this virus is classified as Category B agent by the Centers for Disease Control and Prevention (CDC), much research has been done to generate neutralizing antibodies against it. Antibodies were generated from an immune library from human donors targeting both VEEV envelope glycoproteins E1 and E2 [146]. The isolated Fabs L1A7 and F5 were neutralizing in vitro, with F5 being 300 times more effective than L1A7. Subsequently, F5 was converted into full IgG format and was employed to generate neutralization-escape variants of VEEV for epitope mapping. Within another study, an immune macaque library was used to generate human-like antibodies [119]. One of these antibodies, scFv-Fc ToR67-3B4, was protective in mice when administered 6 h postviral challenge with VEEV Trinidiad strains, showing 80–100% survival after a challenge with 100-fold LD_{50}. However, scFv-Fc ToR67-3B4 was not able to neutralize Trinidad strain in vitro, but other VEEV strains instead, showing that neutralization is not mandatory for an in vivo protective antibody [119]. Another study describes the selection of antibodies from a human naïve scFv gene library using complete, active VEEV particles as antigen. In this case, specific detection of the VEEV strains TC83, H12/93, and 230 by the selected antibodies was proven. Remarkably, none of the selected scFv phage clones showed cross-reactivity with Alphavirus species of the Eastern equine encephalitis virus and WEEV antigenic complex or with Chikungunya virus, making them ideal tools for the immunological detection and diagnostic of Alphavirus species [105]. Two different scFv antibody libraries were constructed from WEEV immunized macaques. Subcloned as scFv-Fc, three antibodies from these libraries specifically bound WEEV in ELISA with little or no cross-reactivity with other alphaviruses and were found to be neutralizing in vitro. In this study, the first antibodies against WEEV, that were shown to be neutralizing in vitro, were developed. About 1 ng/mL of the best antibody (ToR69-3A2) neutralized 50% of 5×10^4 TCID50/mL WEEV [109].

Flu is a disease caused by influenza viruses. In the last years, the bird flu (H5N1) and the pandemic swine flu (a variant of H1N1) moved in the research focus. Due to many genetic events, such as antigenic drift and shift, new influenza variants will occur in the future and will challenge vaccine and diagnostic development [147, 148]. Different groups developed antibodies by phage display against influenza viruses. Sui et al. [104] selected antibodies from a nonimmune scFv library against the H5 hemagglutinin ectodomain. Hemagglutinin is a trimer and the extracelluar part consists of a stalk domain and a globular head domain [149]. They identified ten antibodies binding to the trimeric H5, but not monomeric H5. Interestingly, nine of these antibodies shared the same germline framework (VH1-69). These antibodies were converted into IgG1 and were protective in mice with

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10 or 15 mg/kg in a prophylactic or therapeutic challenge model, respectively. Very remarkably, some antibodies cross-neutralized H1, H2, H5, H6, H8, and H9 influenza strains. These are phage display derived antibodies that are candidates for broad-spectrum influenza immunotherapy [104].

Rabies is caused by the rabies virus that infects the central nervous system. Before Louis Pasteur developed the rabies vaccination, the disease was always fatal. The current post-exposure therapy is based on vaccination—as performed by Pasteur in 1885—and polyclonal anti-rabbies immunoglobulins [150–152]. One hundred forty-seven unique recombinant antibodies against the rabies glycoprotein were selected from two immune scFv libraries [153]. The neutralization of 27 street rabies viruses was tested in vitro and the best neutralizing antibodies were tested in vivo in a hamster rabies infection model. Here, the antibody CR4098 showed 100% postexposure protection with 40 IU/kg [154]. This antibody was further analyzed in combination with an other human antibody CR57, derived by somatic cell hybridization technique [155], in in vitro and in vivo models [152]. The safety of this mAb cocktail named CL184 was tested in a clinical phase 1 study [156] and subsequently in phase 2 studies. The antibodies were named Foravirumab (CR4098) and Rafivirumab (CR57).

An overview of recombinant antibodies generated by phage display against viruses is given in Table 2.

4 Eukaryotic pathogens

Using phage display technology, a large panel of antibodies against a broad range of eukaryotic pathogens has been generated. These antibodies are directed against pathogenic animals, e.g. *Taenia solium* [157], protozoa, e.g. *Cryptosporidium parvum* [158, 159], *Plasmodium falciparum* [160, 161], or *Toxoplasma gondii* [162] and fungi such as *Aspergillus fumigatus* [41]. Not only human pathogens have been in the focus of antibody generation, but also veterinary pathogens like *Myxobolus rotundus* [163] (a fish pathogen) or *Babesia gibsoni* (a dog pathogen) [164] and plant pathogens like *Aspergillus niger* [165], *Fusarium verticilloides* [166], or *Sclerotinia sclerotiorum* [167].

Most antibodies generated in these studies derive from human antibody gene libraries, but libraries from mouse [168], chicken [166], camel [169], or macaque [41] origin have been also successfully applied in phage display to generate monoclonal antibodies against eukaryotic pathogens.

In the following paragraphs detailed examples for the use of phage display for the generation of antibodies against different eukaryotic pathogens are given.

Aspergillus fumigates is the causative pathogen of allergic bronchopulmonary aspergillosis, chronic necrotizing aspergillosis, saprophytic aspergilloma, and highly lethal invasive aspergillosis, the most important *Aspergillus*-related disease [170, 171]. Invasive aspergillosis occurs in immunocompromised individuals for example after hematopoietic stem cell transplantation or solid organ transplantation [172]. Due to its severity, an early diagnosis is crucial for a successful treatment. In 2009, Schütte et al. [41] reported the generation of several antibodies binding specifically to Crf2, an *A. fumigates* antigen of the glycosyl hydrolase family that is located in the cell wall of the growing hyphae. These antibodies could serve as tools for new diagnostic assays such as serum ELISA or histopathological immunofluorescence microscopy. The antibodies described in this study were isolated from two different phage-displayed scFv libraries: One was a macaque immune library with a theoretical diversity of 1.5×10^7 independent clones; while the other was human naïve antibody library HAL4/7 [23]. Both libraries were used in two different panning strategies: the first was performed on recombinant antigen immobilized on immunostrips; the second was carried out in solution with biotinylated antigen. Finally, 16 individual scFv clones were selected, with six clones deriving from the naïve library and ten from the immune library. Interestingly, all antibodies generated on immobilized antigen bind to linear epitopes, whereas all antibodies generated by panning in solution bind to conformational epitopes. Seven of these antibodies bound to their native antigen on growing hyphae of *Aspergillus fumigatus* and did not show cross-reactions to other *Aspergillus* species or *Candida albicans*.

Malaria is a life-threatening protozoal infection of the red blood cells and one of the most common mosquito-borne diseases. Currently, an estimated 3.2 billion people in tropical and subtropical countries live at risk of malaria [173, 174]. In humans malaria is elicited by at least five different species of *Plasmodium*, *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae*, and *P. knowlesi* [175]. *P. falciparum* is responsible for most malaria-related deaths globally, while *P. vivax* is the most widespread parasite [176]. Three different approaches to generate antibodies against *P. falciparum* using phage display are described. In these studies various structures of different developmental stages of the parasite served as antigens. In a first study Roeffen et al. [160] used two scFv immune libraries, constructed from B-lymphocytes from *P. falciparum* patients with transmission-blocking immunity, to generate antibodies against Pfs48/45, a surface protein of *P. falciparum* that is expressed during zygote- and macrogamete stages. Pfs48/45 is a potential vaccine candidate since it is a target of transmission-blocking antibodies that are taken up by the mosquito together with the blood meal and block within the mosquito's intestinal tract the oocyte development [177–179]. The panning was performed on an extract of gametocytes immobilized in immunotubes and the elution was performed by competition with a mixture of four rat monoclonal mAbs, which recognize distinct epitopes on Pfs48/45 (epitopes I, IIb, III, and V). Interestingly, all selected 14 antibody clones bound to epitope III of Pfs48/45. In 2001, Sowa et al. [180] reported the isolation of a human monoclonal antibody against the Block 2 region of *Plasmodium falciparum* merozoite surface protein-1 (PfMSP-1) by phage display from a malaria patient derived scFv library. Lundquist et al. [161] generated a Fab-immune library from leukocytes of 13 adults with acquired immunity to

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Table 2. Recombinant antibodies derived by phage display against viruses

Virus	Target	Library type	Antibody format	Antibody origin	Application	Reference
Blue tongue virus	Complete virus	Semisynthetic	scFv	Chicken	ELISA	[34, 123]
Cucumber mosaic cucumovirus	Complete virus	Semisynthetic	scFv	Human	ELISA, immunoblot	[117]
Dengue	Dengue virus envelope protein E	Naïve	scFv	Human	ELISA, Immunofluorescent assay, in vitro neutralization	[102]
Dengue	Dengue NS5 protein	Naïve	Fab	Human	ELISA, immunoblot, dot blot	[128]
Dengue	Dengue virus envelope protein	Naïve	Fab, IgG	Human, mouse (panel of hybridoma clones)	ELISA, immunoblot, IHC	[141]
Dengue	Dengue NS3	Naïve	Fab	Human	ELISA, in vitro neutralization	[142]
Dengue	NS1 protein	Naïve (nonimmune)	VHH	Llama	ELISA, lateral flow immunochromatograpic assay	[122]
Dengue	n.d.	Immune	Fab, IgG	Chimpanzee	ELISA, immunoprecipitation, in vitro neutralization	[143]
Ebola	Nucleoprotein, envelope glycoprotein, secreted envelope glycoprotein	Immune	Fab, IgG	Human	ELISA, immunostaining, immunoprecipitation, in vitro neutralization	[46]
Ebola	Nucleoprotein	Synthetic	scFv, IgG	Human	ELISA, immunoblot	[220]
Ebola (Zaire)	Viral matrix protein VP40, nucleoprotein	Immune	scFv, IgNAR	Mice, shark	ELISA, immunoblot	[136]
Epstein–Barr virus	LMP1	Naïve	Fab	Human	ELISA, immunoblot, IF, FACS, in vitro inhibition	[221]
Foot-and-mouth disease virus (FMDV)	3ABC	Immune	scFv	Chicken	ELISA, immunoblot	[222]
Grapevine leafroll-associated virus 3	Coat protein	Immune	scFv	Mice	ELISA	[118]
Grapevine virus B	Virus particles	Semisynthetic	scFv	Human	ELISA	[223]
Hantavirus	Nucleoprotein	Immune	VHH	Llama	ELISA, immunoblot	[224]
HCMV	Gycoprotein B and H	Immune	scFv	Human	ELISA, in vitro neutralization	[225]
Hendra and Nipah virus	Attachment envelope glycoprotein G	Naïve	Fab, IgG	Human	ELISA, immunoprecipitation, Immunoblot, in vitro neutralization	[226]
Hepatitis A	Hepatitis A Capsid	Immune	Fab, IgG	Chimpanzee	ELISA, in vitro neutralization	[227]
Hepatitis A	HBsAG	Naïve	scFv	Human	ELISA	[228]
Hepatitis C	NS5B	Naïve	scFv	Human	ELISA, IF, in vitro neutralization	[229]
Hepatitis C	Core, E1, E2	Immune	scFv	Human	ELISA	[230]
Hepatitis C	Core protein	Immune	Fab	Human	ELISA	[231]

(*Continued*)

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Table 2. Continued

Virus	Target	Library type	Antibody format	Antibody origin	Application	Reference
Hepatitis C	E2 glycoprotein	Immune	Fab	Human	ELISA	[232]
Hepatitis E	ORF2 protein	Immune	Fab	Chimpanzee	ELISA, immunoblot	[233]
Herpes simplex virus	HSV1, -2 lysate	Immune	Fab	Human	In vitro inhibition, immuno precipitaton	[234]
Herpes simplex virus	Glycoprotein gD, gB	Presumably immune	Fab	Human	ELISA, immuno precipitation	[235]
Herpes simplex virus	Virus lysate?	Immune	Fab	Human	ELISA, IF	[236]
HIV-1	Integrase	Immune	scFv	Rabbit	ELISA, immunoblot, IF, in vitro neutralization	[38]
HIV-1	gp140	Immune	scFv, scFv-Fc	Human	ELISA, immunoblot, immunoprecipitation, in vitro neutralization	[111]
HIV-1	gp140	Immune	Fab, IgG	Human	ELISA, immunoblot, in vitro neutralization	[237]
HIV-1	gp140	Immune	VHH	Llama	ELISA, in vitro neutralization	[238]
HIV-1	gp120	Synthetic	CH2 domain	Human	ELISA, in vitro neutralization	[129]
HIV-1	p24	Immune	scFv	Mouse	ELISA	[110]
HIV-1	gp41	Synthetic	Fab	Human	Immunoblot, in vitro neutralization	[239]
HIV-1	gp41	Naïve	scFv	Human	ELISA, in vitro neutralization	[240]
HIV-2	gp125 protein	Immune	Fab	Human	ELISA, in vitro neutralization	[241]
Human metapneumovirus	F ectodomain	Presumably immune	Fab	Human	ELISA, IF, in vitro neutralization, in vivo protection	[242]
Infectious haematopoietic necrosis virus	n.d.	Naïve (nonimmune)	scFv	Mouse	ELISA, immunoblot, IHC	[120]
Influenza A	HA (stem region)	Semisynthetic	scFv	Human (IGHV1-69)	ELISA, in vitro neutralization	[116]
Influenza A	HA ectodomain	Naïve	scFv	Human	ELISA, flow cytometry, immunoprecipitation, in vitro neutralization, in vivo protection	[104]
Influenza A	HA (stem region)	Presumably immune	Fab	Human	ELISA, in vitro neutralization	[127]
Influenza A (H5N1)	HA	Semisynthetic	scFv	Human	ELISA	[126]
Influenza A (H5N1)	HA	Naïve	scFv	Human	ELISA, in vitro neutralization, in vivo protection	[243]
Influenza A (H5N1)	HA	Immune	Fab	Chicken	IF, in vitro neutralization, immunoblot	[244]
Influenza A (H5N1)	HA cleavage site	Immune	Fab	Mice	ELISA, IF	[245]
Influenza A (H5N1)	NS1	Naïve	scFv	Human	ELISA, in vitro neutralization, IF	[246]

(*Continued*)

Table 2. Continued

Virus	Target	Library type	Antibody format	Antibody origin	Application	Reference
Japanese encephalitis virus	Domains I,II,III of envelope protein	Immune	Fab, IgG	Chimpanzee	ELISA, immunoprecipitation, in vitro neutralization, in vivo protection	[121]
Japanese encephalitis virus	Envelope protein	Immune	Fab	Human	ELISA, immunoprecipitation, in vitro neutralization	[247]
Measles virus	Virus lysate	Immune (Puumala hantavirus!)	Fab	Human	ELISA, in vitro neutralization	[248]
Norovirus	Norovirus VLPs	Semisynthetic	scFv	Human	ELISA, immunoblot	[249]
Norwalk virus	Norwalk virus VLPs	Immune	Fab, IgG	Chimpanzee	ELISA, FACS, IF, in vitro inhibition	[250]
Poliovirus	Capsid proteins VP1 and VP3	Immune	Fab, IgG	Chimpanzee	ELISA, in vitro neutralization, in vivo protection	[251]
Puumala hantavirus	N protein, G2 protein	Immune	Fab	Human	ELISA	[252]
Puumala hantavirus	Gycoprotein G2	Immune	Fab	Human	ELISA, IF, in vitro neutralization	[253]
Rabies virus	Glycoprotein	Semisynthetic	scFv scFv-Fc	Human	ELISA, immunoblot, in vitro neutralization	[254]
Rabies virus	Glycoprotein	Immune	scFv, IgG	Human	ELISA, flow cytometry, in vitro neutralization	[153, 154]
Rabies virus	n.d.	Immune	Fab	Human	ELISA	[255]
Rabies virus	Glycoprotein (antigenic site II)	Immune	Fab, IgG	Human	ELISA, immunostaining, Immunoblot, in vitro neutralization, in vivo protection	[256]
Rabies virus	Inactivated RABV	Naïve	VHH, VHH pentamer	Lama	ELISA, in vitro neutralization, in vivo protection	[257]
Rotavirus	NSP4	Semisynthetic	scFv	Human	ELISA, immunoblot	[258]
Rotavirus	VP8*	Semisynthetic	scFv	Human	ELISA, immunoblot, in vitro inhibition	[259]
Plum pox virus	NIa protease	Semisynthetic	scFv	Human	Immunoblot, dotblot	[40]
SARS-CoV	S1 domain of spike protein	Naïve	scFv	Human	ELISA, in vitro neutralization	[107]
SARS-CoV	S protein	Immune	Fab, IgG	Human	ELISA, IF, immuno blot, in vitro neutralization	[260]
SARS-CoV	S protein	Immune	scFv	Chicken	ELISA, IF	[261]
Simian immunodeficiency virus	gp120 protein	Immune	Fab	Rhesus macaque	ELISA, in vitro neutralization	[262]
Vaccinia, variola virus	Vaccinia B5 envelope protein	Immune	Fab, IgG	Chimpanzee	ELISA, in vitro neutralization, in vivo protection	[135]

(*Continued*)

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Table 2. Continued

Virus	Target	Library type	Antibody format	Antibody origin	Application	Reference
VEEV	Virus particles	Immune	scFv	Mice	ELISA	[263]
VEEV	E1/E2	Naïve	scFv, scFv-Fc	Human	ELISA, immunoblot, IHC	[105]
VEEV	E1	Immune	scFv, scFv-Fc	Macaque	ELISA, immunoblot, IHC, in vitro neutralization, in vivo protection	[119]
WEEV	n.d.	Immune	scFv, scFv-Fc	Macaque	ELISA, IHC, in vitro neutralization	[109]
West Nile Virus	Domain I and II of WNV envelope protein	Naïve (nonimmune)	scFv	Human	ELISA, in vitro neutralization, in vivo protection	[125]
West Nile Virus	Domain IIII of WNV envelope protein	Immune	Fab	Human	ELISA, IF, in vitro neutralization, in vivo protection (failed)	[264]
White spot syndrome virus	Virus particles	Immune	scFv	Mice	ELISA, in vitro neutralization	[265]
Yellow fever virus	Domain II of envelope protein	Immune	scFv	Human	ELISA,immunoblot, immunoprecipitation, in vitro neutralization	[113]

ELISA, enzyme-linked immunosorbent assays; HA, hemagglutinin; HCMV, human cytomegalovirus; HIV, human immunodeficiency virus; IF, immuno fluorescence microscopy; IHC, immuno histo chemistry; RABV, Rabies virus; SARS, severe acute respiratory syndrome; VLP, virus-like particle; VEEV, Venezuelan equine encephalitis virus; WEEV, Western equine encephalitis virus.

malaria. Three individual Fabs designated RAM1, RAM2, and RAM3 were isolated from this immune library by panning on a recombinant fragment of the merozite surface protein 3 (MSP-$3_{194-257}$). MSP-3 is involved in heme binding, and antibodies against this protein promote eradication of the parasite by monocytes [181, 182]. A synthetic peptide of the N-terminal fragment of MSP-3 is even used in human clinical trials as vaccine [183]. The antibodies were subcloned into IgG1 and IgG3 format for further analysis. Binding of the antibodies to native parasite protein was demonstrated for all three antibodies in immuno blot and immunofluorescence microscopy. RAM1 and RAM2 also bound to their antigen in fixed and permeabilized cells in flow cytometry. The IgG3 format of RAM1 showed in a functional assay (antibody-dependent cellular inhibition assay, ACDI) an inhibition rate that is comparable to affinity-purified polyclonal anti-MSP-$3_{211-237}$ antibodies derived from immune donors. The IgG1 format also showed inhibition in the ACDI, but lower compared to IgG3 [161].

An overview of recombinant antibodies generated by phage display against eukaryotic pathogens is given in Table 3.

5 Antibodies against toxins

Several toxins are classified by the CDC as category A or B agents (for definition see: www.niaid.nih.gov/topics/biodefenserelated/biodefense/pages/cata.aspx) that are relevant for diagnostics and therapeutics. They can easily be disseminated and result in high or moderate mortality rates [184]. In this context, the antibody phage display offers a powerful tool for antibody selection and allows the isolation of neutralizing antibodies against complete active toxins or special domains by using different human naïve antibody libraries with high diversity [185–187]. For toxins, the aim is to find antibodies binding to the cell binding domain of the toxin and neutralize the interaction of this domain with the corresponding cellular target, mainly cell surface proteins. But also antibodies directed against the translocation domain or the enzymatic domain can neutralize the toxicitiy. For the isolation of high-affinity antibodies against specific targets, animals are often immunized with toxoids, nontoxic subunits or selected toxin domains. Well suited are macaques for the construction of immune libraries, because macaque V-genes are very similar to their human counterparts [59, 119, 188–191].

In the following paragraphs, we give detailed examples for antibody generation using phage display and antibody engineering against different toxins.

So far, antibody phage display was successfully used for antibody selection against a panel of toxins classified as category A agents, such as from *Clostridium botulinum* (botulism) [190, 192–194] and *Bacillus anthracis* (anthrax) [59] and also against different category B agents, such as staphylococcal enterotoxin B [195, 196] and ricin toxin from *Ricinus communis* [191, 197]. An example for a high-risk microorganism that

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Table 3. Recombinant antibodies derived by phage display against eukaryotic pathogens

Eukaryotic pathogen	Target	Library type	Antibody format	Antibody origin	Application	Reference
Aspergillus fumigatus	Crf2	Immune/naïve	scFv, scFv-Fc	Macaque/human	ELISA, IF	[41]
Aspergillus niger	Glucose oxidase	Semisynthetic	scFv	Human	ELISA	[165]
Babesia gibsoni	P50	Semisynthetic	scFv	Human	ELISA, IF	[164]
Candida albicans	Als3p and other	Naïve	scFv	Human	ELISA, IF, immunoblot, in vitro neutralization	[266–268]
Cryptosporidium parvum	P23	Semisynthetic	scFv	Human	ELISA	[158]
Cryptosporidium parvum	S16	Semisynthetic	scFv	Human	ELISA	[159]
Fusarium verticillioides	Cell wall-bound proteins	Immune	scFv	Chicken	ELISA, IF	[166]
Fusarium verticillioides	Soluble cell wall-bound proteins	Immune	scFv, scFv-AP	Chicken	ELISA, IF, immunoblot	[269]
Myxobolus rotundus	Spore protein	Immune	scFv	Mice	ELISA, in vitro neutralization	[163]
Plasmodium chabaudi	AMA-1	Immune	scFv	Mice	ELISA, immunoblot	[168]
Plasmodium falciparum	MSP-1	Immune	scFv	Human	ELISA, IF	[180]
Plasmodium falciparum	MSP-3	Immune	Fab, IgG	Human	ELISA, IF, immunoblot, FACS	[161]
Plasmodium falciparum	Pfs48/45	Immune	scFv	Human	ELISA, immunoblot	[160]
Plasmodium yoelii	MSP1	Immune	scFv	Mice	ELISA, immunoblot, in vivo protection	[270]
Sclerotinia sclerotiorum	SSPG1d	Immune	scFv	Mice	ELISA, immunoblot	[167]
Strongyloides venezuelensis	HSP60	Presumably naïve	scFv	Human	ELISA, IF	[271]
Taenia solium	TS14	Immune	VHH	Camel	ELISA, immunoblot	[272]
Taenia solium	*T. solium* metacestodes, peptides	Naïve	scFv	Human	ELISA, IF	[157]
Trypanosoma evansi	Different surface proteins	Immune	VHH	Camel	ELISA, FACS	[169]
Toxoplasma gondii	TgMIC2	Immune	scFv	Mice	ELISA, immunoblot	[162]

ELISA, enzyme-linked immunosorbent assays; IHC, immuno histo chemistry; IF, immuno fluorescence microscopy; FACS, fluorescence-activated cell sorting.

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produces the most toxic substances known with the highest risk of potential use as bioweapons is the Gram-positive, anaerobic, spore-forming bacterium *Clostridium botulinum*, and other *Clostridium* spp. secreting eight different serotypes (A-H) of botulinum neurotoxin (BoNT). Five serotypes (A, B, E, rarely F and only one case of H) are known to cause human botulism, a disease characterized by flaccid muscle paralysis requiring intensive hospital care and passive immunization [198, 199]. Especially, serotype A is recognized as the most toxic substance known, showing LD_{50} of about 1 ng/kg by intravenous route, about 10 ng/kg by the pulmonary route and about 1 μg/kg for the oral route [200]. BoNTs are composed of a disulfide bond-linked 50 kDa light chain and a 100 kDa heavy chain. The heavy chain contains two functional domains (Hc and Hn) that are responsible for toxin uptake into nerve cells by receptor-mediated endocytosis and for the translocation of the light chain across the membrane into the neuronal cytosol. Whereas the catalytic domain of the light chain is responsible for the BoNT toxicity. The current approach for treatment of botulism includes the application of human anti-botulism immunoglobulins, such as BabyBIG, or equine anti-toxin serum. But the human serum stock of BabyBIG is limited and the equine anti-toxin may cause hypersensitivity and serum sickness. In these situations, antibody phage display provides a technology to generate toxin-neutralizing antibodies against each serotype. For instance, a macaque immune library was used to isolate neutralizing scFv with nM affinities against the light chain of BoNT/A [190, 201, 202], but also antibodies against the heavy chain or other relevant serotypes of BoNT are of therapeutic interest. Phage display technology was also used for isolation of single domain antibodies (VHH) after immunization of a llama with a cocktail of 7 BoNT toxoids (A-F) [193]. Another approach was the generation of a human antibody gene library after inducing a BoNT/A-specific immune response by in vitro immunization [194]. Furthermore, antibody phage display was used to generate antibodies against other clostridial toxins such as those from *Clostridium tetani* or *Clostridium difficile* [188, 203].

Anthrax, another serious infectious disease is caused by *Bacillus anthracis*, an aerobic, Gram-positive, spore-forming bacterium that is found in soils around the world. *Bacillus anthracis* secrets two toxins: the lethal toxin (LT) and the edema toxin (ET) [204]. Both are composed of two subunits: the LT consists of the lethal factor (LF), and the protective antigen (PA); while the ET is formed by the edema factor, and PA. It was demonstrated that only LT has an essential role in the pathogenesis of anthrax [205]. The subunit PA is the basis of current vaccines and induces the generation of neutralizing antibodies. In combination with antibiotics, commercial monoclonal antibodies against PA, such as raxibacumab, are commonly used for treatment [206]. In 2012, the FDA approved raxibacumab to treat inhalational anthrax. Due to security issues the use of anti-PA antibodies alone is questionable, since PA could be modified and lose the recognized epitopes while retaining biological activity. An alternative to anti-PA antibodies are antibodies targeting the LF, such as 2LF, which was isolated from an immune library via antibody phage display technology [59]. A combination of an anti-PA antibody with an anti-LF antibody could lead to a synergistic effect and improve the efficacy of the therapy.

An example for bacterial toxins classified as category B agent is staphylococcal enterotoxin B (SEB) from *Staphylococcus aureus*. This bacterium is a potential causative agent for food-borne illness and produces twenty-one types of staphylococcal enterotoxins that cause symptoms of food poisoning such as abdominal cramps, vomiting, and diarrhea [207, 208]. SEB is a single polypeptide of 28 kDa and is the most potent toxin secreted by *S. aureus*. As a superantigen, it stimulates T cells and leads to an overproduction of cytokines, causing clinical symptoms such as fever, hypertension, and in some cases death. Phage display was used to generate recombinant antibodies from a murine immune library [196] and to identify the epitope of a SEB-specific monoclonal antibody using a peptide phage library [209]. Furthermore, a human monoclonal antibody against SEB was isolated from a synthetic human antibody gene library that inhibited SEB binding to MHC-II [195].

The phage display technology was also used to isolate antibodies against ricin, which is also classified as category B agent by CDC. Ricin is a 61-kDa glycoprotein from the castor bean plant (*Ricinus communis*), which consists of two distinct subunits (RTA and RTB). RTB is a galactose- and N-acetylgalactosamine-specific lectin that binds to specific sugar residues on the cell surface, allowing internalization of the toxic RTA by endocytosis [210]. RTA is an RNA N-glycosidase that irreversibly inactivates eukaryotic ribosomes, leading to the inhibition of protein synthesis [211]. Human-like antibodies were selected by phage display from a macaque immunized with RTA. One antibody, 43RCA, had a picomolar affinity and neutralized the biological activity of ricin in vitro [191]. Furthermore, neutralizing VHH with high affinity was selected from a llama immune library. The best antibody C8 was able to neutralize 100% ricin activity in an in vitro assay using 40 μg/mL VHH [197].

In addition to the different toxins that are classified by the CDC as category A or B agents, the number of toxins is almost endless. Different animals are known to produce high potential toxins containing a complex composition. For example, *Tityus serrulatus*, known as Brazilian yellow scorpion and the most dangerous scorpion in Brazil, produces a 61-amino acid peptide, called gamma toxin, which is the major toxic component in the venom [212, 213]. Regarding this toxin, a neutralizing antibody was isolated from a human library via phage display and was capable of protecting mice [185]. The same procedure was used for *Bothrops jararacussu*, a venomous pit viper species endemic in South America. By using a human antibody gene library, different antibodies were selected to inhibit the phospholipase activity of the venom in vitro and reduce the myotoxicity in vivo [214]. Marine organisms can also produce toxins, e.g. the tetrodotoxin (TTX) from pufferfish.

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Table 4. Recombinant antibodies derived by phage display against toxins

Toxin	Species	Target	Library type	Antibody format	Antibody origin	Application	Reference
Androctonus australis venom	*Androctonus australis hector*	AahI' toxin	Immune	VHH, tandem VHH, VHH-Fc	Camel	ELISA, in vivo protection	[273]
Anthrax toxin	*Bacillus anthracis*	Lethal factor (LF)	Immune	scFv	Macaque	ELISA, in vitro toxin neutralization, in vivo protection	[59]
Anthrax toxin	*Bacillus anthracis*	Protective antigen (PA)	Naïve	scFv	Human	In vitro toxin neutralization, in vivo protection	[64]
Bacillus thuringiensis toxin	*Bacillus thuringiensis*	Cry1C d-endotoxins	Semisynthetic	scFv	Human	ELISA, in vitro toxin inhibition	[274]
Bee venom	*Apis mellifera*	Crude venom	Semisynthetic	scFv	Human	ELISA, in vitro toxin inhibition	[275]
Black widow venom	*Latrodectus tredecimguttatus*	Alpha-latrotoxin	Immune	Fab	Mice	ELISA, in vitro inhibition, in vivo protection	[276]
Botulinum neurotoxin	*Clostridium botulinum*	Serotype A - light chain	Immune	scFv	Macaque	ELISA, in vitro toxin inhibition	[201]
Botulinum neurotoxin	*Clostridium botulinum*	Serotype A - light chain	Immune	scFv, scFv-Fc	Macaque	ELISA, immunoblot, in vitro toxin inhibition, ex vivo toxin neutralization	[190]
Botulinum neurotoxin	*Clostridium botulinum*	Serotype A - light chain	Immune	VHH	Camel	ELISA, immunoblot, in vitro neutralization	[277]
Botulinum neurotoxin	*Clostridium botulinum*	Serotype A - heavy chain	Immune	scFv	Murine	ELISA, ex vivo toxin neutralization	[278]
Botulinum neurotoxin	*Clostridium botulinum*	Serotype A - heavy chain	Immune	scFv	Human	ELISA, ex vivo toxin neutralization	[192]
Botulinum neurotoxin	*Clostridium botulinum*	Serotype A - heavy chain	Naïve	scFv	Human	ELISA, ex vivo toxin neutralization	[192]
Botulinum neurotoxin	*Clostridium botulinum*	Serotype A - heavy chain	Immune	scFv	Macaque	ELISA, ex vivo toxin neutralization	[202]
Botulinum neurotoxin	*Clostridium botulinum*	Serotype B - light chain/heavy chain	Immune	scFv, scFv-Fc	Macaque	ELISA, in vitro toxin inhibition, ex vivo toxin neutralization, in vivo protection	[279]
Botulinum neurotoxin	*Clostridium botulinum*	Serotype E - light chain	Immune	scFv, scFv-Fc	Macaque	ELISA, in vitro toxin inhibition, ex vivo toxin neutralization, in vivo protection	[189]
Botulinum neurotoxin	*Clostridium botulinum*	Serotype E - heavy chain	Immune	VHH	Dromedary	ELISA, in vivo protection	[280]
Botulinum neurotoxin	*Clostridium botulinum*	Serotype A/B/C/D/E/F	Immune	VHH	Llama	ELISA, in vitro toxin inhibition	[193]
Brazilian yellow scorpion venom	*Tityus serrulatus*	Gamma-toxin	Naïve	scFv	Human	ELISA, in vivo protection	[185]
C. difficile toxin	*Clostridium difficile*	TcdA	Immune	VHH	Llama	ELISA, immunoblot, in vitro neutralization	[203]

(*Continued*)

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Table 4. Continued

Toxin	Species	Target	Library type	Antibody format	Antibody origin	Application	Reference
C. difficile toxin	*Clostridium difficile*	TcdA, TcdB	Immune	VHH, bispecific VHH	Alpaca	ELISA, in vitro toxin inhibition, in vivo protection	[281]
C. difficile toxin	*Clostridium difficile*	Binary CDT toxin	Immune	VHH, VHH-Fc	Llama	ELISA, in vitro toxin inhibiton, IF	[282]
Enterotoxin B	*Escherichia coli*	EtxB	Semisynthetic	scFv	Human	ELISA, in vitro toxin inhibition	[283]
Fumonisin	*Fusarium Verticillioides*	B1	Semisynthetic	scFv	Human	—	[284]
Jararacussu venom	*Bothrops jararacussu*	Phospholipase A2 (PLA_2)	Naïve	scFv	Human	ELISA, in vitro and in vivo protection	[214]
Mexican scorpion venom	*Centruroides noxius*	Cn2	Naïve	scFv	Human	ELISA, in vivo protection	[285]
Microcystin	*Microcystis aeruginosa*	ADDA	Semisynthetic	scFv	Human	ELISA	[286]
Pit Viper toxin	*Trimeresurus mucros-quamatus*	VSP2	Immune	scFv	Chicken	ELISA, in vitro toxin neutralization, in vivo protection	[287]
Ricin	*Ricinus communis*	Chain A	Immune	scFv	Macaque	ELISA, in vitro toxin neutralization	[191]
Ricin	*Ricinus communis*	Chain A	Immune	VHH	Llama	ELISA, in vitro toxin neutralization	[197]
Shiga toxin	*E. coli* (STEC)	Stx2	Semisynthetic	Fab	Human	ELISA, immunoblot, in vitro neutralization	[288]
Shiga toxin	*E. coli* (EHEC)	Stx1, Stx2	Naïve	scFv	Human	ELISA, FACS, in vitro toxin neutralization	[187]
Shiga toxin	*E. coli* (STEC)	Stx1, Stx2	Immune	VHH	Alpaca	ELISA, in vitro toxin inhibition, in vivo protection	[289]
Staphylococcus enterotoxin B	*Staphylococcus aureus*	SEB	Immune	scFv	Mice	ELISA	[196]
Staphylococcus enterotoxin B	*Staphylococcus aureus*	SEB	Synthetic	Fab	Human	ELISA, immunoblot, in vitro toxin inhibition	[195]
Tetanus	*Clostridium tetani*	tetantus toxoid	Immune	Fab	Macaque	ELISA	[188]
Tetanus	*Clostridium tetani*	tetantus toxoid	Naïve	scFv	Human	ELISA, in vitro toxin inhibition	[186]
Tetrodotoxin (TTX)	*Lagocephalus lunaris*	$C_{11}H_{17}N_3O_8$	Naïve	scFv	Human	ELISA, in vitro and in vivo protection (prolonged survival)	[215]
Tetrodotoxin (TTX)	*Lagocephalus lunaris*	$C_{11}H_{17}N_3O_8$	Immune	scFv	Mice	ELISA	[290]
Thai cobra venom	Naja kaouthia	Neurotoxin	Naïve	scFv	Human	ELISA, immunoblot, in vivo protection	[291]
Vibrio parahaemolyticus hemolysin	*Vibrio parahaemolyticus*	TLH	Immune	scFv	Mice	ELISA, FACS, in vitro neutralization	[292]
Vibrio vulnificus toxin	*Vibrio vulnificus*	VvRtxA	Semisynthetic	scFv	Human	ELISA, in vitro toxin inhibition, IF	[293]

ELISA, enzyme-linked immunosorbent assays.

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Here, scFv were selected from a human naïve antibody gene library neutralizing 99% of TTX activity in vitro [215].

An Overview of recombinant antibodies generated by phage display against toxins is given in Table 4.

6 Conclusion

Antibody phage display allows the generation of recombinant antibodies from different species, including human, llama, camel, macaque, shark, or mice. These antibodies are mainly derived from two types of sources: immune, or naïve libraries. Immune libraries should be preferred when immunized animals or convalescent patients are available, offering the chance to directly isolate affinity matured antibodies. If immunization is not possible or ethically not feasible, naïve antibody gene libraries are an alternative. In such an approach, the antibody generation process is not limited by the immune system. Using antibody phage display a variety of recombinant antibodies was generated for diagnostics and therapy against bacterial, viral pathogens, and eukaryotic pathogens as well as toxins.

We acknowledge funding from the European Community's Seventh Framework Program (FP7/2007-2013) under agreement no. 241832 granted to the AntiBotABE project (http://www.antibotabe.com), funding from Federal State of Lower Saxony, Niedersächsisches Vorab (VWZN2889), and APTDETECT (Eurostars). We thank CNPq for supporting the scholarship of GMSGM. This review is an updated, revised, and enlarged version of a formerly published review [216].

The authors declare that there are no conflicts of interest.

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