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Application of recombinant antibodies for treatment of *Clostridioides difficile* infection: Current status and future perspective

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Clostridioides difficile (*C. difficile*), known as the major cause of antibiotic-associated diarrhea, is regarded as one of the most common healthcare-associated bacterial infections worldwide. Due to the emergence of hypervirulent strains, development of new therapeutic methods for *C. difficile* infection (CDI) has become crucially important. In this context, antibodies have been introduced as valuable tools in the research and clinical environments, as far as the effectiveness of antibody therapy for CDI was reported in several clinical investigations. Hence, production of high-performance antibodies for treatment of CDI would be precious. Traditional approaches of antibody generation are based on hybridoma technology. Today, application of *in vitro* technologies for generating recombinant antibodies, like phage display, is considered as an appropriate alternative to hybridoma technology. These techniques can circumvent the limitations of the immune system and they can be exploited for production of antibodies against different types of biomolecules in particular active toxins. Additionally, DNA encoding antibodies is directly accessible in *in vitro* technologies, which enables the application of antibody engineering in order to increase their sensitivity and specificity. Here, we review the application of antibodies for CDI treatment with an emphasis on recombinant fragment antibodies. Also, this review highlights the current and future prospects of the aforementioned approaches for antibody-mediated therapy of CDI.

KEYWORDS

***Clostridioides difficile*, immunotherapy, hybridoma technology, recombinant antibodies, phage display**

Introduction

Clostridioides difficile, formerly known as *Clostridium difficile*, is a Gram-positive species of spore-forming anaerobic bacteria and it can cause a range of different diseases in humans, such as antibiotic-associated diarrhea (AAD), pseudomembranous colitis (PMC), and toxic megacolon (1, 2). Additionally, *C. difficile* can asymptomatically colonize up to 3% of healthy adults (3). Normally, the bacterium is transmitted by shedding the spores in the environment and it can colonize the gastrointestinal (GI) tract of the infected patients where all of the aspects of *C. difficile* physiology are supported (4, 5). Currently, *C. difficile* infection (CDI) is known as the leading cause of nosocomial diseases associated with antibiotic therapy and healthcare-associated diarrhea in adults (6–8). CDI is characterized by neutrophil accumulation and appearance of distinct plaques (pseudomembranes) in the intestinal lumen (9, 10). It has been suggested that disturbance of the normal gut microbiota as a consequence of antibiotic treatment, could be regarded as a major promoting factor for CDI development (Figures 1A, B). Pathogenesis of *C. difficile* is based on the action of two pro-inflammatory toxins, TcdA and TcdB, that lead to cytoskeleton disintegration, condensation of actin, and eventually cell death (11, 12); these outcomes damage the patient's colonic mucosa and cause severe diarrhea (Figure 1C).

Conventional CDI treatment includes antibiotic therapy that is universally prescribed, especially vancomycin or metronidazole, which are widely used for treating patients with CDI. These antibiotics are non-selective, thus there is a risk of further irritating gut dysbiosis (imbalance of microbiota) and reduction of normal gut commensals, favoring an appropriate niche for *C. difficile* to facilitate its colonization (13). Furthermore, recurrent CDI (rCDI) occurs in approximately 25% of patients and it becomes more prevalent and harder to treat after secondary and tertiary CDI (14, 15). The use of specific anti-*C. difficile* antibiotics, such as fidaxomicin, an antibiotic that has been approved by the Food and Drug Administration (FDA), is recommended for reducing the adverse effects of nonselective antibiotic therapy; for instance, fidaxomicin reduced the relapse rate compared to vancomycin, however, high costs of fidaxomicin restricted its application (16).

With the emergence of rising resistance to antibiotics, the need to design effective treatments for *C. difficile* is urgently highlighted. One of the alternative approaches to antibiotic treatment is antibody-mediated therapy, including bezlotoxumab (the first and only FDA-approved human monoclonal antibody (mAb) to prevent rCDI) (17), mAb cocktails (18, 19), polyclonal antibodies (IGIV: immune globulin intravenous) (1, 20, 21), and active vaccination (22, 23) that can be effective for the treatment or prevention of CDI (24). Antibody therapy for CDI has many advantages over using conventional antibiotics, the most important of which is maintaining the intestinal microbial balance, composition and diversity (Figure 1D) (25). For instance, metronidazole is generally active on anaerobic bacteria like *Bacteroides* and *Bifidobacterium* (26), and vancomycin have also shown antimicrobial activity against *Bacteroides* and *Enterococcus* spp., also fidaxomicin, despite it is known as a specific anti-*C. difficile* agent, possesses antibacterial activity against *Bacillus* spp., *Bifidobacterium*, *Enterococcus* spp., and *Lactobacilli* (28). Accordingly, antibiotic-mediated gut dysbiosis can facilitate the germination of *C. difficile* spores in the patient's gut, which may consequently lead to induction of rCDI cycle (29, 30). Moreover, the end of each course of traditional antibiotic therapy for CDI is regarded as the main period of patients' vulnerability to CDI relapse (31). Notably, even if the over-mentioned disadvantages related to antibiotic therapy are ignored, treatment with antibiotics are not sufficiently effective in the treatment of rCDI and also the prevention of *C. difficile* spread (32, 33). Thus, development of efficient antibody-based therapies can allow to preserve the microbiota integrity and also reducing CDI recurrences. In recent decades, antibodies have been widely used as tools for basic research (34), diagnosis (35–40), and therapy (41–45). At present, the recombinant antibodies (rAbs) technology helps to develop more cost-effective antibodies (46–48). Here, we comprehensively discuss the latest research contributed to rAbs technology and the precise role of rAbs in the antibody-mediated therapy for CDI.

Abbreviations: AAD, Antibiotic-associated diarrhea; Cwps, Cell wall proteins; *C. difficile*, *Clostridioides difficile*; CDI, *C. difficile* infection; cDNA, Complementary DNA; CROPs, Combined repetitive oligopeptide structures; CDRs, Complementarity determining regions; EMA, European Medicines Agency; Fabs, Fragment antigen binding domains; Fc, Fragment crystallizable; FACS, Fluorescence-activated cell sorting; FDA, Food and Drug Administration; FliC, Flagellin; FliD, Flagellin filament cap proteins; FR, Framework regions; FMT, Fecal microbiome transplantation; GI tract, Gastrointestinal tract; GTD, Glucosyltransferase domain; IC50, Half maximal inhibitory concentration; HAMA, Human anti-mouse antibody reaction; HIV-1, Human immunodeficiency virus type 1, HuMAbs, Human monoclonal IgGs; Ig, Immunoglobulin; IGIV, Immune globulin intravenous; mAbs, Monoclonal antibodies; NGS, Next-generation sequencing; PaLoc, Pathogenicity locus; PD-1, Programmed cell death protein 1; pAbs, Polyclonal antibodies; PMC, Pseudomembranous colitis; RA, Rheumatoid arthritis; rAbs, Recombinant antibodies; rCDI, Recurrent CDI; SLP, S-layer proteins; scFv, Single-chain fragment variable; scRNA-seq, Single-cell RNA sequencing technologies, sdAb, Single-domain antibodies; SoC, Standard of care; TLRs, Toll-like receptors; TNFα, Tumor necrosis factor α; VEGF-A, Vascular endothelial growth factor A; V_H, Variable regions of heavy domain; V_L, Variable regions of light domain.

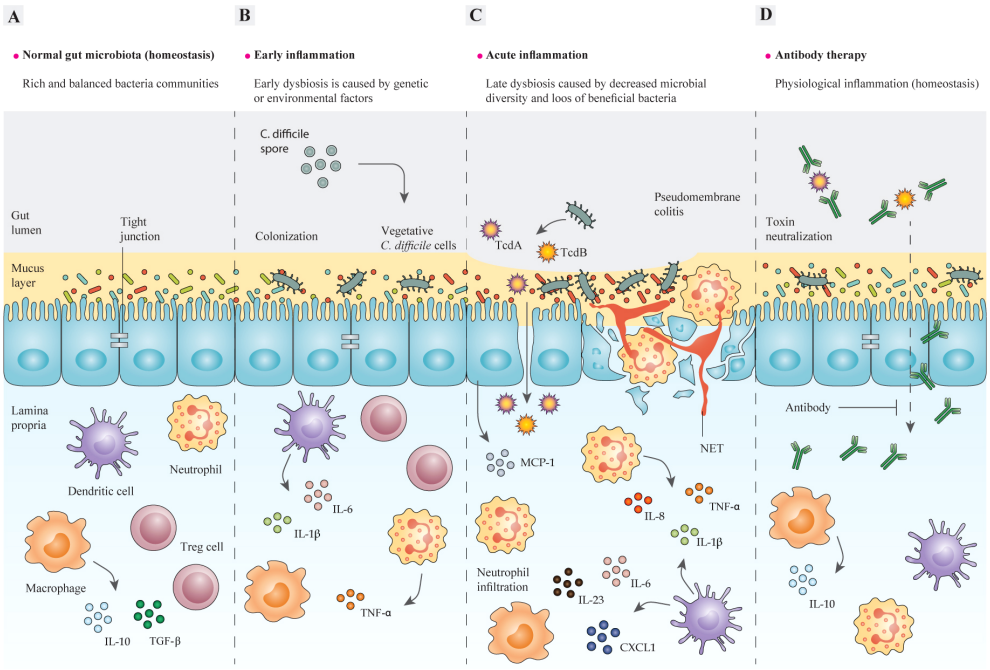

FIGURE 1
The structure of the intestinal epithelium in different stages of *C. difficile*-induced inflammation. **(A)** In the steady-state, intestinal bacteria are segregated from epithelial cells (IECs) by an intact mucosal layer. A well-balanced relationship is maintained between intestinal microbiota and mucosal barriers during gut homeostasis, so that gut microbes and host immune cells can mutually communicate to regulate the functions of intestinal epithelium. Commensal bacteria and pathogens can modulate the intestinal immune response to release various chemokines and cytokines, or inhibit their production, such as IL-8 and MCP-1. In a homeostasis state, pathogen recognition leads to inducing antigen-presenting cells (APCs), like macrophages, dendritic cells (DC), and neutrophils, which produce pro-inflammatory cytokines like IL-1β and IL-23. In contrast, commensals can stimulate APCs to promote anti-inflammatory cytokines, like IL-10 and Treg responses, which suppress the immune response by inhibiting cytokine production, therefore homeostasis and self-tolerance are maintained. In some cases, the interactions between commensal bacteria and the gut epithelium may lead to the discharge of TGF-β from macrophages, which triggers fibroblast proliferation (tissue remodeling). **(B)** The imbalance between mucosal barriers and gut microbes is promoted by the dysfunction of mucosal barriers, including decreased production of mucin that causes intestinal inflammation. A combination of genetic and environmental factors especially antibiotic administration, leads to gut microbiota dysbiosis, and thereby enrichment of pathobionts and susceptibility to *C. difficile* may occur. The adherence of *C. difficile* to the epithelium activates host inflammatory response via different signaling pathways, which result in production of inflammatory cytokines. **(C)** Epithelium colonization and toxin production by *C. difficile* act on colonic epithelial and immune cells as inflammatory stimuli and induce tissue damage. In particular, the cytopathic effects of TcdA and TcdB lead to disruption of the tight junctions, which causes toxins to cross the epithelial barriers and further induce inflammatory cytokine production in lymphocytes, macrophages, and DCs. This further contributes to inflammation and neutrophil influx, which subsequently results in a pseudomembrane formation, which is characteristic of *C. difficile* colitis. **(D)** The application of monoclonal antibodies (mAbs) developed for targeting toxins of *C. difficile* helps modulate direct damage to the colonic epithelium caused by toxins and restores the homeostatic immune responses and ameliorates inflammation. IECs, intestinal epithelial cells; IL-8, interleukin 8; MCP-1, monocyte chemoattractant protein-1; APCs, antigen-presenting cells; IL-1β, interleukin 1 beta; IL-23, interleukin 23; Treg, regulatory T cells; IL-10, interleukin 10; TGF-β, transforming growth factor-β; TNF-α, tumor necrosis factor alpha. IL-6, interleukin 6; DC, dendritic cell; TcdA, Toxin A; TcdB, Toxin B; mAbs, monoclonal antibodies.

Recombinant antibody technologies

The typical antibody molecule contains three functional components, two fragment antigen binding domains (Fabs) and the fragment crystallizable (Fc) (Figure 2) (49). The antigen recognition process is accomplished by the six complementarity determining regions (CDRs) that make up antigen-binding region (paratope), and are located at the tips of the Fabs, highlighted in the variable regions of heavy and light domains (V_H and V_L, respectively); namely, CDRH1, CDRH2, CDRH3, CDRL1, CDRL2, and CDRL3 (50). An antibody-antigen interaction is formed by foundation of non-covalent forces between the paratope of antibody and the antigenic determinant (epitope) of antigen, which also determine the affinity of antibodies. Notably, the length and composition of the CDR sequences are highly variable, especially in the CDR3 (51–54).

Since the last decades, polyclonal antibodies (pAbs) and mAbs have been used for diagnostic and therapeutic purposes, so-called theranostics applications (55–58). The function of antibodies is based on their high affinity and specific binding to target molecules, which make them reliable therapeutic/

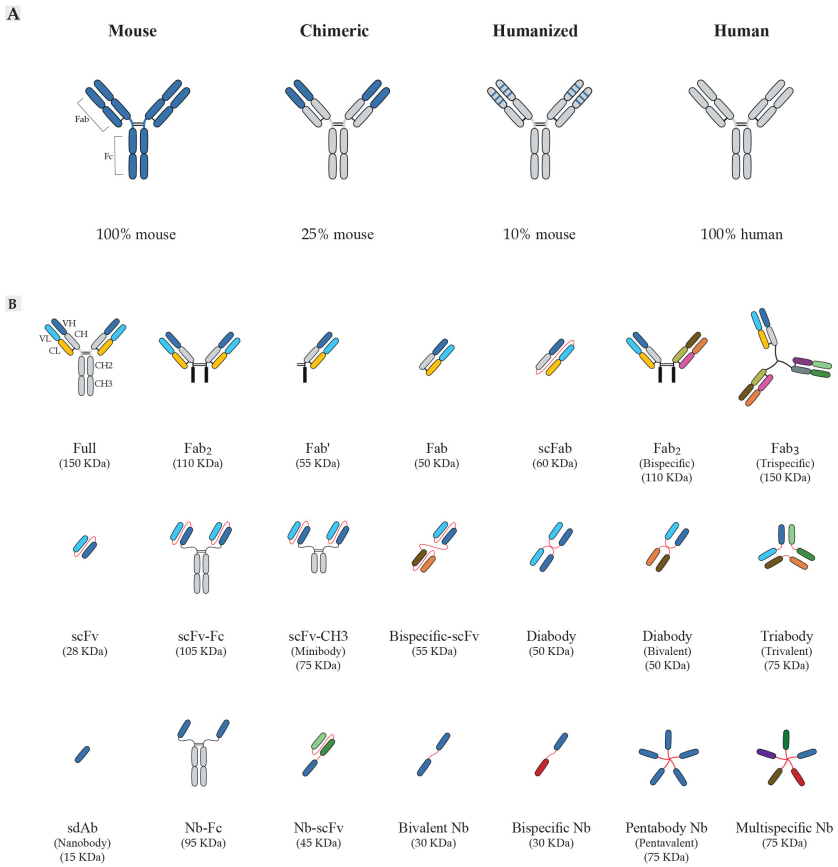

FIGURE 2
Schematic overview of antibody formats. **(A)** Antibody humanization from murine antibodies (blue domains) to fully human antibodies (gray domains). Chimeric antibodies are formed by fusing sequences of murine variable domain **(V)** regions to human constant **(C)** regions. Humanized antibodies are generated by grafting sequences of murine CDR to the human V-framework regions and expressed with human C regions. **(B)** Representation of recombinant antibody fragments. The design of small recombinant antibodies based on the VH and VL domains of the parental mAbs is provided by antibody engineering. Here, the basic types of antibody fragments including Fab, scFv, and sdAb, and their derivatives are represented. Fabs are composed of VL and a constant CL linked to VH and CH1 by a disulfide bond between the CL and CH1 domains. There are other formats based on Fabs, including Fab´ composed of a structure similar to Fab; scFabs composed of VL and CL linked to VH and CH1 by a flexible glycine-serine linker (Gly4Ser)3; bispecific Fabs fragments composed of 2 × Fab fragments joined by a disulfide bond; trispecific Fab fragments composed of 3 × Fab fragments joined by a disulfide bond with the paratope specificity for more than one antigens. scFvs are composed of VL linked to VH by a flexible glycine-serine linker (Gly4Ser)3. scFvs can be engineered to generate multivalent or multi-domain structures, including scFv-Fc composed of scFv to the Fc region of the antibody; scFv-CH3 composed of scFv to the CH3 region of the antibody; diabodies composed of 2 × scFv fragments joined by the shortening of the linker peptide; bispecific-scFv composed of 2 × scFv fragments joined by a flexible glycine-serine linker (Gly4Ser)3; forms of multimeric scFv composed of multiple scFv fragments with the paratope specificity for more than one antigens. sdAbs or nanobodies (Nb) composed of VH and devoid of the VL chain completely. The examples of other sdAb formats engineered are Nb-Fc composed of sdAb to the Fc region of the antibody; Nb-scFv composed of sdAb to scFv fragment of the antibody; bivalent Nb composed of 2 × Nb fragments; bispecific Nb composed of 2 × Nb fragments with the different paratope specificity; multispecific Nb composed of multiple Nb fragments with the different paratope specificity for more than one antigens. mAbs, monoclonal antibody; CDRs, complementarity-determining regions; CH, constant domain of the heavy chain; CL, constant domain of the light chain; VH, variable domain of the heavy chain; VL, variable domain of the light chain Fc, fragment crystallizable region; Fab, fragment of antigen-binding; scFv, single-chain fragment variable; sdAb, single-domain antibody; Nb, nanobody.

diagnostic tools (57, 59). In this regard, the hybridoma technology, known as mAb production technique, has been widely used for the production of antibodies with desired characteristics (60). Although the hybridoma technology has been applied successfully in numerous cases, it has many shortcomings. Most antibodies provided by hybridoma are murine antibodies (100% mouse protein), limiting their therapeutic applications due to human anti-mouse antibody reaction (HAMA) (61). However, this problem can be modified by manipulating the sequence and structure of the recombinant antibody technologies for production of chimeric antibodies, humanized antibodies, use of transgenic animals (i.e. XenoMouse technology), and surface display technologies (62–64). These methods are briefly described below.

Chimeric, humanized, and fully human antibodies derived from mice

Cloning of immunoglobulin (Ig) gene segments was first performed in 1977 (65) and after that, in 1984, chimeric antibodies were produced by fusing the antigen-binding variable domains of a mouse mAb to constant domains of human antibodies (Fc fragment), which is composed of approximately 75% human and 25% mouse protein (66, 67) as presented in Figure 2A. Some advantages of chimeric antibodies are as follows: they retain the specificity of the parental mouse antibodies, and they have very low immunogenic properties for antibody administration in clinical trials (59), and longer half-lives compared to the parental antibodies (68). Successful performance of these antibodies shown by many studies, has led to their widespread use in diagnostics or therapy for several diseases (59, 61).

The first report on humanized antibodies was published in 1986. Humanized antibodies were then constructed by grafting the gene segments of the CDRs of mouse-sequence origin into the human framework regions with highest similarity to the original mouse framework (Figure 2A). This results in an antibody with 90% human and 10% mouse amino acid sequence (69, 70). Although the specificity of these antibodies was similar to mAbs, their affinity was lower. This can at least be partially overcome by different methods like secondary directed mutagenesis after humanization (71). Humanized antibodies are used for treatment of various diseases including cancers, autoimmune disorders, and infectious diseases (61, 72–75).

Transgenic technology for antibody production was first suggested by Alt et al. (1985), while Green et al. (1994) for the first time reported the generation of mice with germline modifications (76, 77). In transgenic mice, human Ig loci is introduced into the mouse genome instead of the respective murine counterpart (78), and immunization of mice with a specific antigen leads to activation of B lymphocytes producing human antibodies. The mAb isolation approach in this technique is similar to hybridoma, except that the entirely human antibody is produced in established cell lines. Production of completely humanized antibodies has led to widespread use of this method for therapeutic purposes (59, 79).

Recombinant antibody fragments

Other strategies that overcome the disadvantages of hybridoma, e.g. being time-consuming and inefficient to generate antibodies toward toxic antigens, are techniques developed based on *in vitro* antibody generation. These techniques, known as surface display technologies, were invented concomitant to the application of humanized antibodies. The generation of antibodies by display technologies (e.g. mammalian cell display, ribosome display, yeast display, and phage display) is based on the interaction of displayed antibody fragments with an antigen *in vitro*. For this approach diverse antibody gene libraries are used (48, 80). Typically, the recombinant antibody libraries are constructed from different sources, including B cell repertoire derived from an immunized donor (immune libraries) (81, 82), pre-immune repertoires (naive libraries) (81, 83, 84), and synthetic design (synthetic libraries) (85, 86). Immune libraries are usually constructed to select a specific antibody against a target antigen in medical research (87) and exhibit more advantages than naive display libraries, including the increased likelihood of selecting antibodies with high affinity/stability, and *in vivo* affinity maturation (88).

An advantage of display technologies is its ability to achieve specific antibodies, which can be improved along with recombinant DNA technology and antibody engineering through cloning and expression of a variety of antibody fragments in different expression systems such as bacteria, yeast, and mammalian cells (48, 89–91). Furthermore, complementary DNA (cDNA) encoding antibodies are stable in this method and can be stored for several years (49). In display technologies, the size of the antibody molecule is reduced and only the intact antigen-binding site (paratope) is preserved, such as fragment antigen-binding (Fab), single-chain fragment variable (scFv), and single-domain antibodies (sdAb) (46, 49) as presented in Figure 2B. These antibody fragments can eventually improve the antibody's therapeutic properties in terms of better penetration in tumor tissue, faster blood clearance for imaging purposes, lower retention times in non-target tissue, and inferior immunogenicity. The affinity and specificity of rAbs are similar to conventional antibodies, thus no avidity effect exists (49, 92). In addition, *in vitro* expression systems for production of antibody fragments can help to achieve sufficient amounts of antibodies for diagnostic and therapeutic purposes (49, 93, 94). Since these technologies depend on *in vitro* screening rounds, they do not involve the immune system (94). Another point is that application of rAb system allows to construct new recombinant proteins such as bivalent antibodies (95, 96), multivalent antibodies (97–99), and bispecific antibodies (100), for different purposes.

A unique feature of rAbs is their high affinity and specificity for target molecules. This feature has led to their successful use in therapeutic grounds, so that different antibody fragments have been generated by rAb techniques and successfully applied in different fields (93, 94, 101). There are more than 500 antibodies under clinical investigations worldwide for various diseases like autoimmunity and inflammation, cancer, organ transplantation, infectious diseases, diabetes, arthritis, and hypercholesterolemia, and as antidote against several toxins (61, 102–104).

The popular types of antibodies produced by display technologies are Fab, scFv, and sdAb (92). In the past two decades, scFv technology has been widely used for targeting

haptens (105, 106), proteins (107, 108), carbohydrates (109, 110), receptors (111, 112), tumor antigens (113), and viruses (114), and they showed good potential in therapeutic and diagnostic applications. Currently, based on Therapeutic Antibody Database (http://tabs.craic.com/) more than 100 scFv antibodies are in human clinical trials, a few of which, blinatumomab (anti-CD19, CD3), brolucizumab (anti-VEGF-A), avelumab (anti-hPD-L1), belimumab (anti- BAFF, BLyS), darleukin (anti-L19-IL2), tralokinumab (anti-IL-13), briakinumab (anti-IL-12, IL-23), and atezolizumab (anti-PD-L1) are approved by FDA.

In parallel, successful application of Fabs has been shown against different diseases; for example, ranibizumab is a humanized mAb Fab derived from phage display library and it was approved in 2006 for inhibiting vascular endothelial growth factor A (VEGF-A) (115).

Recently, sdAb, named VHH or nanobody, have received considerable attention and often are produced by constructing antibody libraries from immunized animals such as camel, llama, or alpaca (116). The major advantage of sdAbs is their high capacity to penetrate specific target cells, e.g., solid tumors, diseased or infected cells (117). Importantly, sdAbs can easily be constructed as bispecific or multispecific formats which have multiple functions (116, 118–120); however, the main disadvantage of these formats of antibodies is their short half-life and rapid elimination from circulation (121). Furthermore, the sdAbs have been applied as high potential tools for different purposes, including treatment of infectious and inflammatory diseases (120, 122, 123), and detection of toxins such as cholera toxin (CT) (124).

Surface display platforms

As mentioned above, different surface platforms, e.g., mammalian cell display, ribosome display, yeast display, and phage display, are used for screening recombinant antibody libraries and each has different advantages and disadvantages (48, 89). The common method used in different platforms is the same for displaying the libraries and contains several rounds of screening for selecting specific antibodies (48). The main differences in platforms are related to the differences in the diversity of libraries, different efficiencies in screening, and the possibility of antibody maturation (48). In particular, yeast and mammalian displays can increase the affinity maturation of antibodies due to the ability of post-translational modifications in displayed fragments (48, 89, 125). Moreover, the main advantage of these platforms is their compatibility with fluorescence-activated cell sorting (FACS), leading to precise control over screening and selecting high-affinity antibodies, which bind specifically to the targeted antigen (125, 126). However, multivalent binding has been reported as a significant drawback of yeast or mammalian cell platforms because of the presence of antibody density on their cell surfaces. This disadvantage allows for isolating antibodies with lower affinity compared to monovalent techniques, e.g., phage display (48). Additionally, the construction of large libraries is challenging in these platforms, as far as the number of unique antibody copies in the yeast or mammalian libraries is several orders of magnitude lower than in phage libraries, leading to the selection of few candidates in the panning process (89, 127).

The phage, ribosome, and bacterial display libraries have been constructed with more than 10^{11} clones, allowing for the generation and screening of a huge diversity of clones (48, 86, 128). Antibody expression in these systems, especially in phage display, is considered a straightforward process with many advantages (95, 96). Moreover, since gene manipulation in these platforms is easy, the generation of antibodies with higher affinity and specificity is more feasible than other recombinant technologies (49, 129). Notably, over the past decades, phage display has been the most widely used system for the production of rAbs and was awarded 2018 Nobel Prize in Chemistry. Application of phage antibody libraries has been reported for identification of various targets, including haptens, and foreign and self-antigens (106, 130, 131). Moreover, the use of phage display for therapeutic purposes has received great attention. The first FDA-approved therapeutic antibody produced based on this technique was adalimumab (Humira), that was approved as an anti-tumor necrosis factor α (TNFα) human antibody against rheumatoid arthritis (RA) in the US in 2002 (86, 132). Overall, 15 therapeutic antibodies isolated from phage display libraries have been approved by the US FDA (Table 1), and over 110 antibodies are currently in clinical evaluation stage (http://tabs.craic.com/).

Although phage display has many advantages, the main limitation of this method, which is also seen in the ribosome and bacterial display techniques, is that antibody interrogation is based only on the binding properties of binders and not on functional features (147–149). Moreover, until recently, these technologies were unable to preserve the cognate V_H–V_L pairing, because obtaining paired information requires determining the antibody sequence at individual cell level (150, 151). This limitation sometimes leads to rising antibodies with inferior selectivity and weaker biophysical features compared with human antibodies or Igs (152), however, this disadvantage can be adjusted in immune libraries due to the pre-enrichment and *in vivo* affinity maturation (88).

Over the last decade, single-cell technologies combined with next-generation sequencing (NGS) approaches have been introduced as a high-throughput screening of antibodies, which are highly boosted by microfluidic platforms (153, 154). Surface display platforms combined with single-cell technologies can provide open-source computational tools to interrogate more in-depth V_H–V_L sequences in display libraries (150, 155) and allow screening of diverse antibodies based on their functional properties, affinity, and biophysical characteristics (151). In the following, single-cell RNA sequencing (scRNA-seq) technologies are discussed briefly.

TABLE 1 FDA-approved human mAbs derived from phage display libraries.

Product name	Trade name	Fragment antibody	Final antibody format	Target	Marketing company	Application	Approval	Reference
Adalimumab (D2E7)	Humira	scFv	IgG1-κ	TNFα	AbbVie	Rheumatoid arthritis	2002	(133)
Ranibizumab	Lucentis	Fab	Fab	VEGFA	Roche/ Genentech	Macular degeneration growth factor A	2006	(134)
Belimumab	Benlysta	scFv	IgG1-λ	BLyS	GSK	Systemic lupus erythematous	2011	(135)
Raxibacumab	Abthrax	scFv	IgG1-λ	Anthrax PA	GSK/HGSI	Inhalation anthrax	2012	(44)
Ramucirumab	Cyramza	Fab	IgG1-κ	VEGFR2	Eli Lilly	Gastric cancer, colorectal cancer and non-small cell lung cancer	2014	(136)
Necitumumab	Portrazza	Fab	IgG1-κ	EGFR	Lilly/ AstraZeneca	Squamous non-small cell lung cancer	2015	(137)
Atezolizumab	Tecentriq	–	IgG1-κ	PD-L1	Roche	Metastatic lung cancer, Renal cancer	2016	(138)
Ixekizumab	Taltz	Fab	IgG4-κ	IL-17A	Eli Lilly	Psoriasis	2016	(139)
Guselkumab	Tremfya	Fab	IgG1-λ	IL-23, subunit p19	Janssen Biotech	Plaque psoriasis	2017	(140)
Avelumab	Bavencio	Naive Fab	IgG1-λ	PD-L1	Serono/Pfizer	Merkel cell carcinoma, metastatic urothelial carcinoma	2017	(141)
Lanadelumab	Takhzyro	Fab	IgG1-κ	pKaI	Dyax Corp.	Hereditary angioedema attacks	2018	(142)
Emapalumab	Gamifant	scFv	IgG1-λ	IFNγ	NovImmune SA	Primary hemophagocytic lymphohistiocytosis	2018	(143)
Moxetumomab pasudodox	Lumoxiti	scFv	Murine IgG1 dsFv	CD22	MedImmune/ AstraZeneca	Hairy cell leukemia,	2018	(144)
Caplacizumab	Cablivi	Nanobody	Humanized V_H-V_H	VWF A1 domain	Sanofi/Ablynx	Acquired thrombotic thrombocytopenic purpure	2018	(145)
Tralokinumab	Adtralza	scFv	IgG4-λ	IL-13	AstraZena	Atopic dermatitis	2021	(146)

TNF-α, tumor necrosis factor-alpha; VEGFA, vascular endothelial growth factors A; BLyS, B-lymphocyte stimulator; PA, protective antigen; VEGFR2, vascular endothelial growth factor receptor 2; EGFR, epidermal growth factor receptor; PD-L1, programmed death-ligand 1; IL-17A, interleukin 17A, IL-23, interleukin-23; pKaI, plasma kallikrein; IFN-γ, interferon gamma; CD22, cluster of differentiation-22; vWF-A1, A1 domain of von Willebrand Factor (vWF).

Single-cell RNA sequencing technologies (scRNA-seq)

Over the last decade, the application of NGS approaches to explore antibody repertoire has been extensively investigated, some of these approaches can recover the cognate V_H-V_L pairing and identify the genotype of antibodies (150, 152, 156–158). However, there is a need for a system that can support high-throughput phenotype-genotype screening (159, 160). Recently, single-cell platforms have been applied to probe the diversity of V_H-V_L chains of antibodies and to assess the specificity of antibodies at large scales (150, 152, 153, 157). These technologies can maintain the genotype and phenotype linkage of antibodies and allow them to be compatible with high-throughput RNA sequencing output (155). The basic steps for scRNA-seq include sample collection, single-cell isolation and capture, cell lysis, barcoded reverse transcription, cDNA amplification sequencing, bioinformatics analysis, and library preparation (Figure 3). The most widely used techniques for single-cell isolation and capture include FACS, microfluidic system, and magnetic-activated cell sorting (154).

Droplet-based microfluidics is one of the most efficient technologies for single-cell studies and has shown great potential for functional high-throughput screening, genomics studies, and comprehensive analysis of the immune repertoire diversity (147, 159). These technologies have conceptual advantages, such as portability of equipment, consuming just a few cell numbers for performing large-scale studies, high separation efficiency, high degree of automation, fast analysis, and enabling the preservation of phenotype-genotype linkage within the droplet (159, 161). These advantages make droplet microfluidics a preferable tool for biomedical research and single-cell studies (150, 155). In these systems, B cells are encapsulated into droplets by a co-flow emulsion droplet microfluidic chip for mRNA capturing, and then heavy and light chains of the antibody in a single cell are characterized by a hydrogel bead coupled with uniquely barcoded primers (154, 159). Subsequently, the barcoded cDNAs are sequenced and exploited for generating recombinant antibody libraries, e.g., phage display and yeast display libraries (Figure 3) (150, 152, 162).

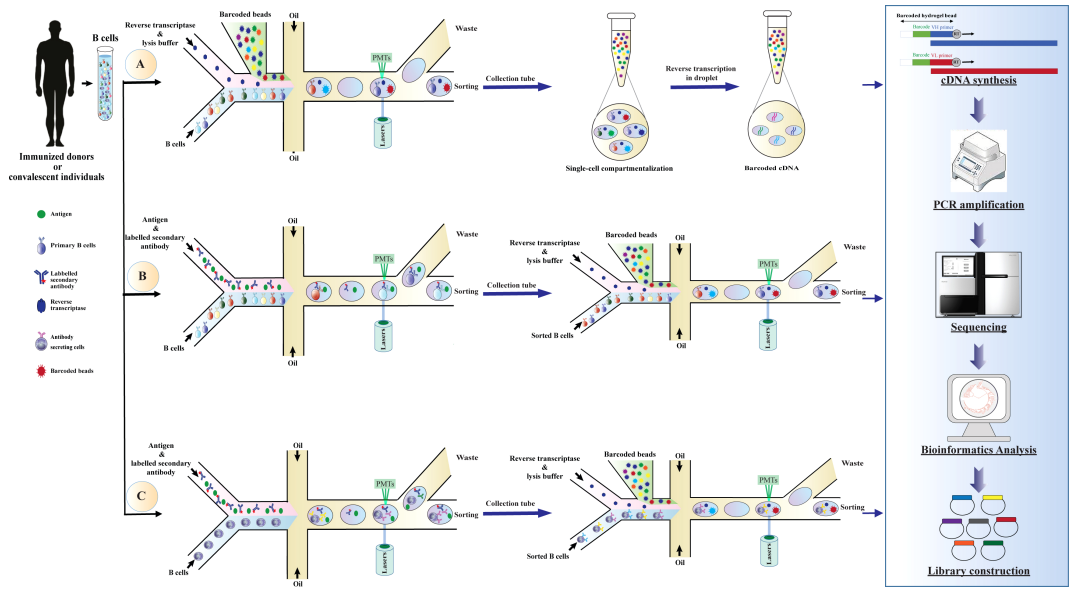

FIGURE 3
Schematic overview of application droplet-based microfluidics for high-throughput screening of human B cells repertoire and library construction. Development of antibody libraries is based on screening **(A)** human B cells repertoire, **(B)** B cells binding to a specific antigen, or **(C)** B cells secreting antibodies binding to a specific antigen. The basic steps of the method are as follows: droplets with a specific fluorescence intensity are deflected into the collection channel through electric fields. The selected cells are re-compartmentalized in the droplets simultaneously with a hydrogel bead coupled with uniquely barcoded polyT primers to generate droplets containing a single cell and a single bead. After cell lysis, reverse transcription is conducted for the V_H and V_L domains in the droplets. Since the cDNAs synthesized from each cell are conjugated with a unique barcode (corresponding to mRNAs produced in the droplets), cognate V_H and V_L pairs can be identified by NGS and subsequently used for library generation. cDNA, complementary DNA; mRNA, messenger RNA; V_H, variable domain of the heavy chain; V_L, variable domain of the light chain, NGS, next-generation sequencing; PMTs, photomultiplier tubes.

In this way, microfluidic technology can be applied for library construction from B cell repertoire derived from immunized donors or convalescent individuals, supporting native chain-paired library generation and direct screening of antibodies (Figure 3A). This method can easily check millions of primary human B cells derived from the native repertoire into an automated and sensitive screening platform (150, 152). Recently, applications of this technology for constructing several libraries have been reported, including coupling recombinant repertoires obtained from the microfluidic platform with phage display technology to rapidly screen specific antibodies for various targets such as influenza hemagglutinin (150) and programmed cell death protein 1 (PD-1) (162). Also, droplet-based microfluidics combined with yeast display technology are exploited for selecting neutralizing antibodies for human immunodeficiency virus type 1 (HIV-1), Ebola virus glycoprotein, and influenza hemagglutinin (152). This method can be applied as a powerful tool for screening high-affinity antibodies for therapeutic approaches (147, 163, 164).

Additionally, droplet-based microfluidics can be used for high-throughput single-cell screening for the selectivity of antibodies unique to each B cell in the presence of the target antigen. This system can be applied for the screening of primary B cells or Ig-secreting cells such as plasma cells obtained from immunized donors (28) or plasmablasts from human peripheral blood (159, 165, 166). In these systems, B cells have been co-cultivated with antigen-expressing cells or antigen-coated beads in the presence of fluorescently labeled secondary antibodies. B cells or antibodies secreted from them can bind to the antigen and generate a specific fluorescence intensity. Specific B cells can be identified based on intensity fluorescence peak and used for constructing specific libraries (154) as presented in Figures 3B, C. The successful development of these technologies has been reported in several studies (157, 159, 160). This technology facilitates the selection of high-affinity antibodies with distinct functional features for therapeutic purposes and can help construct libraries with high specificity and functional activities (154, 162). Taken together, single cell technology based on microfluidic systems can be integrated with display techniques or applied to generate libraries containing matured and specific antibodies with high diversity and affinity.

Development of recombinant therapeutic antibodies for *C. difficile*

Previous studies on the pathogenicity of *C. difficile* have confirmed that its major virulence factors are the large secreted

glucosyltransferase protein toxins TcdA and TcdB, which are encoded within a 19.6-kb pathogenicity locus (PaLoc) (167, 168). TcdA and TcdB are involved in the development of inflammatory response associated with the production of chemokines and cytokines, neutrophil influx, fluid secretion, and cell damage and death (6, 169). In addition to the above-mentioned toxins, some more virulent strains produce a binary toxin or *C. difficile* transferase (CDT), which is an actin-specific ADP-ribosyltransferase (170). Other factors including cell wall proteins (Cwps that mainly act as adhesions), flagellin (FliC), flagellin filament cap protein (FliD), and S-layer proteins (SLP) are also involved in the pathogenicity of *C. difficile* that contribute to colon localization, and evasion of the immune system surveillance (171). These factors may play a role in the initiation of bacterial pathogenesis trough inducing inflammatory responses and interactions with toll-like receptors (TLRs) (169). Furthermore, several extracellular enzymes that are produced by *C. difficile* may be important in normal physiological processes and survival of the bacterium in the GI tract, however, their decisive role in pathogenesis is still uncharacterized. Noteworthy, typical clinical symptoms of CDI can only be caused by strains producing TcdB, or both TcdB and TcdA (172). Moreover, in many studies, it is proven that the major virulence factor in CDI is TcdB, which can alone induce severe organ damage *in vivo* (6, 168, 173), but it should be noted that the virulence features are also retained in strains expressing only TcdA (12, 174, 175). Nevertheless, the abundance of $TcdA^{-}B^{+}$ strains isolated from patients is higher than $TcdA^{+}B^{-}$ strains (176, 177). According to this, considerable efforts have been made to use antibodies that can directly target toxins instead of the bacterium, thus, both TcdA and TcdB can be used as promising target antigens in antibody production (178–180).

Antitoxin A/B antibodies

Over the past 30 years, several antibodies have been developed against TcdA or TcdB, and their effectiveness has been evaluated in animal models, and some of them have shown therapeutic value in CDI treatment. A summary of the antibodies used in the study of *C. difficile* infection is presented in Table 2. Among these antibodies, the C-terminal domain of both TcdA and TcdB, i.e., building combined repetitive oligopeptide structures (CROPs), which is involved in the binding of toxins to carbohydrate receptors on the surface of host cells, have been mostly used as target antigens for antibody production (12). Based on several previous studies, immunization of animal models using the CROPs of *C. difficile* toxins could induce production of toxin neutralizing antibodies among challenged animals with either toxins or live bacteria (222–225). The application of different antibodies, e.g. pAbs, mAbs, and rAbs, has been widely investigated in CDI treatment. Due to the effectiveness of serum therapy in the treatment of several bacterial diseases (226), this type of therapy was also suggested for CDI. Several studies have investigated the therapeutic efficacy of pAbs derived from animals that were immunized with mutants of TcdA and TcdB (181, 227), and the results showed the effectiveness of this method in reduction of CDI incidence in infected animals. Furthermore, the use of serum antibodies against TcdA and TcdB was suggested for patients with CDI (20, 228). In this regard, the application of a polyclonal ovine antibody that binds to toxins and neutralizes their effects has been successfully applied for CDI treatment. Interestingly, this method did not exhibit an immunogenic effect on the patients (189). Moreover, serum antibodies derived from healthy blood donors have been investigated in many studies (229–231), some of these studies demonstrated that this method can be successfully used as an oral treatment in patients with severe CDI who were refractory to standard treatments (232). Recently, bovine antibodies from hyperimmune colostral milk can be regarded as a powerful orally-administered drug candidate that is currently in the clinical development (190, 227). However, efforts to produce more effective therapeutic antibodies are continued since antibodies must have a clear advantage over other available treatments to be accepted as a promising agent by clinicians and regulatory authorities.

The earliest application of specific mAbs for *C. difficile* toxins in animal research was reported by Lyerly et al. (1986). This work showed that pre-mixing mAbs and oral administration of their admixture can completely protect hamsters against detrimental effects of toxin (194). So far, several antibodies have been generated against *C. difficile* and their effectiveness has been evaluated in various models of animals. Some of these studies proposed the use of anti-toxin antibodies as beneficial agents for *C. difficile* treatment, particularly for preventing the initial episodes of CDI and reducing the risk of rCDI (1, 182, 233). Most investigations on anti-toxins are based on antibody production from animal sources, while the clinical application of such antibodies requires humanization to reduce the potential immune-related adverse events (irAE) for human use. Presently, there is only one FDA-approved antibody to treat rCDI (bezlotoxumab), and the other antibodies are in the clinical or laboratory stages, which are discussed below.

Bezlotoxumab: The first FDA-approved therapeutic monoclonal antibody for the prevention of CDI recurrence

The first specific human anti-toxin mAbs for TcdA and TcdB were reported by Babcock et al. in 2006, and today, they are known as actoxumab and bezlotoxumab, developed against TcdA and TcdB, respectively. In this work, human monoclonal IgGs (HuMAbs) were produced in transgenic mice immunized with inactivated TcdA, TcdB, and recombinant TcdB as antigens (204). Screening of various hybridomas *in vitro* and *in vivo* led to

Table 2 Summaries of antibodies used in the study of *Clostridioides difficile* infection.

Antibody type	Antibody name	Target	Antibody source	Neutralizing *in vitro*	Protective *in vivo*			Summary	Reference (s)
					Animal model	Ab administration route	Protection		
Polyclonal antibodies	Bovine IgG	Culture filtrate	Cow colostrum	N/d	Hamster	Oral	Yes	Hyperimmune IgG fraction from bovine colostrum protected hamsters with high efficacy.	(181)
	Bovine IgG	Inactivated TcdA, culture filtrate	Cow colostrum	Yes	Rat	Orogastric dosing	Yes	Bovine colostrum IgG neutralized the effects of TcdA or culture filtrate (TcdA and TcdB).	(182)
	IgY	rTcdA/ rTcdB	Chicken	No	Hamster	Oral	Yes	Antibodies that bind to the C- terminal domain of toxins were the most effective. Anti-TcdA alone was able to protect ~70% hamsters, but the antibody combination of anti-TcdA and anti-TcdB protected ~100% hamsters.	(183)
	Anti-TcdB	rTcdA/ rTcdB	Mouse	N/d	Hamster	I.P.	Yes	Serum antitoxin antibodies mediate systemic and mucosal protection from *C. difficile* disease in hamsters.	(184)
	Bovine colostrum	culture filtrate	Cow colostrum	N/d	Hamster	Oral	Yes	Orally Administration colostrum IgG for 3 days conferred ~90% protection to hamsters. Recurrent CDI were not observed in any patients.	(185)
	IgG	SLP	Rabbit	Yes	Hamster	Orogastric dosing	Yes	Anti-SLP antibodies increased survival compared with control groups and modulated the course of CDI.	(186)
	IVIG	–	Human immunoglobulin	N/d	Human	Oral	Yes	Human immunoglobulin protected patients. It was highly effective in treating patients with multiple recurrences of CDI.	(187)
	IgY	FliC, FliD, Cwp84	Chicken	Yes	Hamster	Oral	Yes	FliD-specific IgY significantly protected hamsters from CDI.	(188)
	IgG	TcdA/TcdB	Sheep	Yes	Hamster	I.P.	Yes	Administration of anti-TcdA sera reduced the symptom severity but conferred no protection to hamsters against death. The combination of anti-TcdA and anti-TcdB protected ~50 to 90% hamsters after administration of 75 mg of each antibodies.	(189)
	HBC	spores, vegetative cells, rTcdB	Cow colostrum	Yes	Mouse	Oral	Yes	Administration of TcdB-specific colostrum prevented CDI in Mouse. Coadministration TcdB-specific colostrum with spore or vegetative cell-targeted colostrum reduced recurrence rate up 67%.	(190)
	Bovine colostrum	TcdA/TcdB	Cow colostrum	N/d	Hamster	Oral	Yes	Bovine colostrum protected ~50 to 100% hamsters. In addition to efficacy to treat primary CDI, it prevented of recurrent CDI in infected hamsters.	(191)
	OraCAb	rTcdA/ rTcdB	Sheep	Yes	Hamster	Oral	Yes	The OraCAb neutralized toxin production in *in vivo* and *in vitro*. Coadministration of OraCAb together with vancomycin prevented simulated CDI recurrence in hamsters.	(192)
	Secretory IgA (sIgA)	TcdA/TcdB	Human	N/d	Hamster	Oral	Yes	Coadministration of human secretory IgA (sIgA) and vancomycin enhanced survival in hamsters challenged with *C. difficile*.	(193)
Monoclonal antibodies	PCG-4 IgG	TcdA	Mouse	No	Hamster	Oral	Yes	PCG-4 neutralized the effects of TcdA in *in vivo*. It binds to 2 epitopes of C-terminus of TcdA and blocks the binding of TcdA to Caco-2 cells.	(194) (195)
	G-2 IgG	TcdA/TcdB	Mouse	No	Hamster	Oral	No		

(Continued)

10.3389/fimmu.2022.972930

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Antibody type	Antibody name	Target	Antibody source	Neutralizing *in vitro*	Protective *in vivo*			Summary	Reference (s)
					Animal model	Ab administration route	Protection		
								G-2 binds to a shared epitope on TcdA and TcdB, but it did not neutralize either toxin.	(196) (194) (197)
	37B5 IgG	TcdA	Mouse	No	Rabbit Mouse	I.P.	No	37B5 neutralized the effects of TcdA in a rabbit ligated ileal loop assay, however, it did not protect mouse challenged with TcdA.	(198)
	A9, 141-2, C11	TcdA	Mouse	N/d	Mouse	I.V.	Yes	Antibodies Recognized C-teminal domin of TcdB. I.V. administration of antibodies protected Mouse from the effects of *C. difficile*.	(199)
	3358, 3359	TcdA (CROPs)	Mouse	N/d	Hamster	I.P.	No	Modestly neutralizing mAbs observed. The use of mAbs cocktail showed a better effect in neutralizing the toxin. Overall, there are no protection observed.	(200)
	A1H3	TcdA	Mouse	N/d	Piglet	–	N/d	A1H3 enhanced cell-surface recruitment of TcdA.	(201) (202)
	1G3, 1B5, 2D4, 2C7, 4A4, 5D8	TcdA (CROPs)	Mouse	Yes	Mouse	I.P.	Yes	A mixture of 4A4 and 5D8 was useful for detection of and protection against TcdA. 4A4 protected. 50% mouse challenged with purified TcdA, while in combination with 2C7 increased up ~90% protection. There is no protection by 5D8.	(203)
XenoMouse antibodies	Actoxumab (CDA1)	TcdA (CROPs)	Transgenic mouse (human IgG1)	Yes	Mouse Hamster Piglet Human	I.P.	Yes	A mixture of two actoxumab and bezlotoxumab was able to protect animal models (mouse, hamster, piglet, and human). No/poor efficacy observed to protect piglet/human when actoxumab used alone.	(18, 204–207)
	Bezlotoxumab (MK6072, CDB1, MDX-1388, 124-152)	TcdB (CROPs)	Transgenic mouse (human IgG1)	Yes	Mouse Hamster Piglet Human	I.P.	Yes	A mixture of two bezlotoxumab and actoxumab was able to protect animal models (mouse, hamster, piglet, and human). bezlotoxumab alone was capable of efficacious to reduce recurrence CDI rate in human.	(18, 204, 206, 207)
	A2, B1, B2	rTcdA/ rTcdB	Transgenic mouse (human IgG1)	Yes	Hamster	I.P.	Yes	A mixture of A2 (anti-TcdA) with B1 and B2 (anti-TcdB) neutralized TcdA and TcdB in *in vitro* assays. 3-Mabs cocktail antibodies reduced mortality rate and disease severity in hamsters challenged with *C. difficile*.	(208)
Humanized antibodies	PA-50, PA-41	TcdA/Tcd, GTD	Mouse	Yes	Hamster	I.P.	Yes	Both antibodies neutralized of TcdA and B from multiple C. difficile ribotypes. A mixture of two PA-50(anti-TcdA) and PA-41 (anti-TcdB) protected~90 to 100% hamsters until day 39 postinfection.	(202, 209, 210)
	CA997	TcdA (CROPs)	mAb (humanized IgG1)	Yes	Hamster	I.P.	Yes.	CA997 neutralized TcdA from multiple *C. difficile* ribotypes. The combination CA997 with CA1125 and CA1151 protected hamsters with more potency than actoxumab/bezlotoxumab combination.	(19)
	CA1125, CA1151	TcdB (CROPs)	mAb (humanized IgG1)	Yes	Hamster	I.P.	Yes	A mixture of both mAbs neutralized TcdB. In combination with CA997 had Greater protection than actoxumab and bezlotoxumab combination.	(19)
	CANmAbA4, CANmAbB4, CANmAbB1	rTcdA/ rTcdB	Mouse	Yes	Hamster	I.P.	Yes	The humanized anti-TcdA (CANmAbA4) and anti-TcdB (CANmAbB4 and CANmAbB1) antibodies neutralized both toxins in *in vitro*. Coadministration	(211)

(Continued)

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Antibody type	Antibody name	Target	Antibody source	Neutralizing *in vitro*	Protective *in vivo*			Summary	Reference (s)
					Animal model	Ab administration route	Protection		
								of CANmAbA4 and CANmAbB4 protected ~ 85% hamsters after administration of 50 mg/kg of each antibody.	
Recombinant antibodies		TcdB	Human scFv	Yes	–	–	N/d	The scFv Fragment had high specificity for toxin B and no cross-react observed with non-toxigenic strains of *C. difficile*.	(212)
	A4.2, A5.1, A19.2, A20.1, A24.1, A26.8	TcdA (CROPs)	Llama VHH	Yes	–	–	N/d	VHH fragments neutralized TcdA. The mixture of VHH had more efficacy for toxin neutralization. Administration of *B. longum* transformed with A20.1 or A26.8 resulted in gut expression. There is No data for infection models.	(213–216)
	B5.2, B13.6, B15.5, B39	TcdB (CROPs)	Llama VHH	No	–	–	N/d	Anti-TcdB VHH were non-neutralizing *in vitro*. No data for infection models.	(213, 215)
	B4, B5, B12, B17	TcdB (CROPs)	Human V_L	No	–	–	N/d	–	(213)
	ABA (AH3–E3–E3–AA6)	TcdA/TcdB	Alpaca VHH (bispecific, tetrameric)	Yes	Mouse	I.P.	Yes	Tetramer VHHs neutralized TcdA and TcdB in *in vitro*. Tetramer protected mice challenged with TcdA/B and *C. difficile*.	(217)
	B2, E2, G3, D8	TcdB: CROPS	Llama VHH	Yes	Hamster	Oral	Yes	VHHs (B2, G3, and D8) neutralized TcdB, while combinations of VHHs did not improve neutralizing potency. Administration of *Lactobacilli* displaying B2 and G3 resulted in gut expression. An administration model with *Lactobacilli* displaying B2 and G3 VHHs was protective.	(218)
	Anti-SLP	SLPs	Llama VHH	Yes	–	–	N/d	Anti-SPL fragments strongly bonded to different ribotypes of *C. difficile*.	(219)
	Anti-FLiC, Anti-FliD	FliC/FliD	Human scFv	Yes	–	–	N/d	scFv antibodies was strongly able to detect *C. difficile* 630 and also to inhibit bacterial motility.	(220)
	Anti-TcdB	TcdB	Human scFv, scFv-Fc	Yes	–	–	N/d	The epitopes of the neutralizing and non-neutralizing scFv fragments were identified and a new neutralizing epitope within the glucosyltransferase domain of TcdB was recognized.	(221)

C. difficile, *Clostridioides difficile*; mAb, monoclonal antibody; scFv, single-chain fragment variable; VHH, variable domain of heavy-chain antibody or nanobody; IgY, egg yolk antibodies; IVIG, intravenous immunoglobulin; HBC, hyperimmune bovine colostrum, SLP, surface layer proteins; FliC, flagellin Protein, FliD, flagellar capping protein; Cwp84, a surface-associated protein; LPS, bacterial lipopolysaccharides; CROPS, receptor-binding domain; GTD, glucosyltransferase domain; N/d, not determined; IP, intraperitoneal; IV, intravenous.

10.3389/fimmu.2022.972930

the isolation of specific antibodies against TcdA (CDA1) and TcdB (MDX-1388) that could significantly reduce hamster mortality in primary CDI treatment and CDI relapse models.

The evaluation of the safety and pharmacokinetic of HuMAb CDA1 confirmed that this antibody is safe at doses between 0.3 and 20 mg/kg. Interestingly, the combination therapy of HuMAb CDA1 and antibiotics could significantly decrease the hamster's mortality (204). The high efficacy of these two antibodies in the protection of animals against CDI led to the initiation of the first human clinical trial for treating CDI by using CDA1 and MDX-1388, as far as, the placebo-controlled phase III trials, MODIFY I and II, were done to determine the efficacy of both antibodies in CDI patients (17, 234). After that, anti-TcdA (CDA1) and anti-TcdB (MDX-1388) were named actoxumab and bezlotoxumab, respectively. These clinical trials showed that actoxumab does not have clinical efficacy in clinical phase III (MODIFY II), while bezlotoxumab reduced CDI recurrence from ~40% in this phase (absolute rate reduction of ~10%) (17).

The safety and pharmacokinetic studies of bezlotoxumab confirmed the acceptable performance of this antibody. However, it should be noted that the assessment reports of the FDA and then the European Medicines Agency (EMA) demonstrated that administration of bezlotoxumab in subjects with baseline congestive heart failure, increased heart failure incidence and all-cause mortality compared to placebo-treated patients. Finally, in 2016, bezlotoxumab (Zinplava©) was approved by the FDA and EMA, for the prevention of rCDI in adult patients (≥18 years) (U.S. Food and Drug Administration, 2016 (https://www.fda.gov/). Notably, bezlotoxumab can only reduce the rate of CDI relapse to ~40% compared to placebo and is unfavorable for treating acute CDI (Navalkele and Chopra, 2018). Therefore, bezlotoxumab can be applied as an effective therapy for preventing rCDI. However, the clinical effectiveness of the drug should be assessed in further studies (235).

Current international guidelines advocated the standard of care (SoC) antibiotics for CDI treatment, and metronidazole or vancomycin are recommended for mild to moderate CDI, and fidaxomicin for severe disease and/or multiple CDI episodes accordingly (13, 14, 27, 32, 236). Among the proposed antibiotics, fidaxomicin is the only specific antibiotic for *C. difficile*, however, there is no study to date that has compared the cost-effectiveness of fidaxomicin with bezlotoxumab. The only cost-effectiveness analysis is related to the comparison of the effect of fidaxomicin with standard therapy plus bezlotoxumab as reported by Lam et al. focusing only on rCDI (237). Additionally, it is proven that fidaxomicin plus bezlotoxumab has similar effect to other SOC antibiotics (i.e., vancomycin or metronidazole) plus bezlotoxumab (42, 238, 239). Notably, pharmacoeconomic analyses demonstrate that standard therapy plus bezlotoxumab could be cost-effective compared with standard therapy alone, especially in preventing of rCDI episodes in those >65 years of age, those with severe CDI, and immunocompromised patients (234, 237, 240–243). The results of a retrospective study on high-risk patients treated with bezlotoxumab have proved the clinical effectiveness of this antibody, in which 73% of the treated patients remained free of recurrence during a follow-up of three months (241). Additionally, bezlotoxumab administration to immunocompromised patients could prevent rCDI in 71% of these patients (243). Therefore, the adjunctive use of bezlotoxumab can be recommended in those patients with three or more risk factors promoting recurrence development.

Toxin-neutralizing antibody that are in clinical phases

In addition to bezlotoxumab, some other antibodies are currently in clinical trial phases and some have been considered for oral use, thus, the number of such antibodies as promising therapies may increase in the future. For example, humanized murine toxin-specific mAbs reported by Marozsan et al. (2012), PA-50 (humanized anti-toxin A mAb), and PA-41 (humanized antitoxin B mAb), were found as an acceptable choice for treating CDI and can be used as a non-antibiotic agent for improving CDI management (209). Additionally, in this work the authors showed that the application of a mixture of both PA-50/PA-41 in hamster models of CDI led to 95% long-term survival compared to 0% survival of animals treated with the standard antibiotic vancomycin. Also, the efficacy of the combination of PA-50/PA-41 was much higher than that of the combination of actoxumab and bezlotoxumab. Interestingly, the potency of PA-50 was significantly higher than actoxumab *in vitro*, because of its multivalent interactions with TcdA. PA-41 is also significantly more potent compared to bezlotoxumab. Evaluation of the efficiency of these mAbs against different ribotypes (RTs) of *C. difficile in vitro*, e.g., RT001, RT002, RT003, RT012, RT014, RT017, RT027, and RT078, revealed their broad neutralizing activity; such properties introduce these mAbs as attractive candidates in non-antibiotic therapy of CDI (209). As shown in this study, a combination of antibodies could increase the effectiveness of antibody therapy. The combination of antibodies directed against different target structures/epitopes/domains may have a synergistic effect, thus increasing the performance of antibody therapy (244). This hypothesis was examined and proven in other studies (19, 208, 211). Importantly, some clinical trials demonstrated that the combination of a cocktail of anti-toxins A and B antibodies and antibiotics like vancomycin, significantly decrease the recurrence rate compared to antibiotic therapy alone for CDI (1, 18, 234, 242). This indicates great potential of co-administration of anti-toxin antibodies and antibiotics over standard antibiotic therapy.

Recombinant fragment antibodies neutralizing toxin A/B

In addition to humanized antitoxin and mouse mAbs under development and their combination application, rAb fragments can be used as alternative immunotherapeutic agents for treating CDI. In the previous part, we described the advantages of using rAb fragments. Today, rAb technology is known as a rapid and high-performance approach to introduce the next generation of immunotherapy agents. The rAb fragments can be applied as a suitable option to bind epitopes that are inaccessible for conventional antibodies due to their high tissue penetration capability (245, 246). Additionally, the small size of the rAb allows them to access immuno-silent cavities in enzymes and receptors (247). These properties of rAbs have made them favorable agents for *C. difficile* toxin neutralization, which may have even greater efficacy in the GI tract (213, 248).

As mentioned earlier, scFv antibodies have applied to neutralize potent toxins. However, there are few publications about the isolation of scFv antibodies that bind *C. difficile* toxins. Deng et al. (2003) reported the isolation of toxin B-neutralizing scFv from a scFv library by phage display technology; however, the work did not progress beyond binding assays (212). Recently, antibodies targeting different domains of TcdB were isolated (221). After conversion to the IgG-like bivalent scFv-hFc format 31 humans, anti-TcdB antibody fragments were further characterized for TcdB binding and neutralization. Analysis of the epitopes of these antibody fragments to identify neutralizing and non-neutralizing epitopes was done using domain mapping, TcdB fragment phage display, and peptide arrays. These results provided new insights into the function of different toxin regions and their relevance to neutralization and toxicity because a new epitope for toxin neutralization within the N-terminal glucosyltransferase domain (GTD) of TcdB was identified. The bivalent scFv-Fc formats have been constructed and characterized in several studies (96, 249), their results showed the conversion of antibody candidates into an IgG format like bivalent scFv-Fc is recommended for further validation and characterization of selected fragments. Moreover, it seems that scFv-Fc formats provide rapid screening of many candidate antibodies, thus it is preferable over full IgG format (49, 244, 250).

Similarly, the application of sdAbs for treating toxin-mediated diseases has been assessed by several groups (251, 252), and antitoxin sdAbs were also successfully generated to neutralize *C. difficile* toxins in recent studies (180, 213, 253). The first isolation of specific sdAb fragments for detecting *C. difficile* toxins was reported by Hussack et al. (2011 and 2012). In this work, specific llama VHHs against CROPs fragments from each toxin were isolated from an immune phage display library. The results showed that six of seven selected anti-TcdA sdAbs inhibited the cytotoxicity of TcdA when added at 1000 nm concentration, whereas none of the seven selected anti-TcdB sdAb fragments blocked TcdB cytotoxicity. Interestingly, the mixture of anti-TcdA sdAbs improved toxin neutralization at lower sdAb concentrations (213, 248). In another work, Murase et al. described the isolation of sdAbs binding to CROPs of TcdB (215). Antibody fragments isolated in this study demonstrated different binding properties, and some of them e.g. B5.2, B13.6, and B15.5, despite having high binding affinity, were incapable of toxin neutralization *in vitro*. One of the isolated sdAb, B39, seemed to have four binding sites for TcdB. Interestingly, B5.2 cross-reacted with the TcdA but was unable to neutralize it.

Noteworthy, an attractive alternative to the combination of antibodies is the use of bispecific or multispecific antibody constructs, which can be easily prepared by rAb technologies (254–256). This approach was previously reported for the design of therapeutic antibodies against several diseases such as cancers, inflammatory and infectious diseases (257, 258). Concerning *C. difficile*, Yang et al. (217) characterized bispecific sdAb antibodies with high efficiency that target both toxins and can effectively treat severe CDI. This work developed several sdAbs that recognized and neutralized either TcdA or TcdB. Additionally, this research group designed a novel construct consisting of multiple-antitoxin sdAbs (AH3–E3–E3–AA6), later called ABA, which neutralized both TcdA and TcdB and reduced disease symptoms in a mouse model of CDI. Evaluation of the efficiency of ABA in the neutralization of the toxin from different clinical *C. difficile* isolates indicated considerable ability of ABA to neutralize toxins in the isolates that produced both TcdA and TcdB, but inefficient to neutralize the toxin from $TcdA^{-}B^{+}$ *C. difficile* strains. Importantly, the application of ABA could protect mice against a systemic challenge of a mixture of TcdA and TcdB, indicating its high potency also *in vivo*. The results of this study have proven high therapeutic efficacy of sdAb antibodies against both toxins in multivalent or bispecific formats, through reducing the morbidity and mortality associated with this disease.

In the work reported by Hussack et al. (2018), the stability of sdAb fragments isolated was also increased through fusions to Fc to reach the neutralizing potency of bezlotoxumab in *in vitro* assays. Epitope binding revealed that bivalent sdAb-Fc fusions can target TcdB at regions both similar and distinct from the bezlotoxumab binding sites so that some constructs of sdAb-Fcs could recognize the sites distinct from the binding site of bezlotoxumab and other sdAb-Fcs had binding site similar to bezlotoxumab. Overall, the sdAbs described in this work were effective in toxin B neutralization when provided in bivalent sdAb-Fc formats (259).

The advantage of rAbs is the possibility of their genetic manipulation to increase their efficiency. Sulea et al. (2018) considered an affinity maturation platform to construct a set of mutant sdAbs neutralizing TcdA. These results supported the role of mutation in enhancing the affinity of antibodies. In this

regard, the development of double-mutant T56R and T103R neutralized TcdA cytotoxicity with a half maximal inhibitory concentration (IC50) of 12 nM and enhanced sdAb affinity to toxin A (260).

Probiotic bacteria expressing antitoxin fragments

Recently, the use of probiotic bacteria expressing antibodies has attracted the attention of many researchers. For *C. difficile*, the expression of antibodies on the surface of probiotic bacteria has been investigated in some studies and it was shown that this approach retained neutralizing potency of the antibodies used. In this regard, Andersen et al. (2015) assessed the expression of four anti-toxins sdAb fragments on the surface of *Lactobacillus paracasei* strains. Interestingly, two strains of the probiotic delayed the death of hamsters challenged by *C. difficile* spores, whereas no animal in the control group survived (non-sdAb expressing *Lactobacillus* group vs infection only group). Additionally, 50% of the hamsters receiving the probiotic survived until the end of the experiment. Noteworthy, following administration of purified anti-TcdB VHH alone, no protection was observed *in vivo* that was probably related to the degradation of the antibodies in the GI tract. In fact, the expression of antibodies on the surface of *Lactobacilli* helps preserve antibodies in the GI tract (218). Also, Shkoporov et al. (2015) described the expression of two sdAbs on the surface of *Bifidobacterium longum* and showed TcdA neutralization *in vitro*. Moreover, administration of recombinant *B. longum* to mice revealed *in vivo* expression of both sdAbs in the gut of mice (261). Recently, Chen et al. (2020) assessed the expression of a single tetra-specific antibody on the surface of *Saccharomyces boulardii*. The results showed that engineered probiotic bacteria neutralized both toxins and demonstrated that it can protect mice in both primary and rCDI models. This study proposed that the co-administration of engineered *S. boulardii* with antibiotics may have potential to be regarded as a therapeutic tool for patients with CDI (262). Based on these results, the application of engineered probiotic bacteria producing surface-exposed anti-toxins can be considered a complementary approach for CDI future treatment.

Recombinant antibodies to other *C. difficile* targets

In addition to TcdA and TcdB, CDT is an important pathogenicity factor of *C. difficile* that can cause death in animals (263). Unger et al. (2015) introduced and characterized specific sdAbs from phage display libraries generated from immunized llamas. These sdAbs could block enzymatic and cytotoxic activities of CDT. Thus, these sdAbs can be considered as a promising new tool for diagnosis and therapy of CDI (253).

Noteworthy, administration of antitoxin antibodies is effective only in developed CDI and does not prevent the initial *C. difficile* colonization step, thus antibodies that are capable of binding to other virulence factors of *C. difficile* appear attractive as complement tools for many researchers. For example, antibodies against cell-surface components involved in the adherence to host gut tissues and colonization, such as SLPs (264, 265), flagella (266), Cwp84 (267), are other promising complementary targets for antibody therapy. Several studies suggest that the application of anti-SLPs antibodies can be a good choice for CDI treatment (268, 269). For instance, anti-SLP sdAbs could inhibit bacterial motility in *in vitro* assays (219). The sdAbs demonstrated broad binding specificity to different *C. difficile* RTs, including RT001, RT027, RT012, RT017, RT023, and RT078. These results showed that targeting SLPs with rAbs should be considered in antibody-mediated therapy for CDI.

Several studies have also proposed the use of anti-flagella antibodies as therapeutics (268, 270). There is one report on the isolation of scFv fragments against FliC and FliD of *C. difficile* that inhibited bacterial motility (220). According to this, it seems that targeting surface components of *C. difficile* by antibodies can be of therapeutic value, thus targeting components such as FliC and FliD, Cwp84, and PSII polysaccharides could be appraised in the development of antibodies in the future.

Discussion

Today, the use of antibodies for therapeutic purposes has received much attention. In this regard, obtaining antibodies with specificity, sensitivity and high affinity has always been a challenge for researchers. Recently, many efforts have been made to achieve specific antibodies against *C. difficile* proteins for therapeutic purposes, and a considerable amount of research is still ongoing in this area. It has been proven that rAb fragments, especially sdAbs are potentially effective tools for therapy of *C. difficile* (271, 272). In most cases, rAbs have many advantages, including the ability to be genetically modified to improve selectivity, sensitivity and immobilization, thus they have been proposed as alternatives to conventional antibodies.

Recently, rAbs have been widely studied for clinical applications. Antibody therapy has been considered in GI diseases as an efficient method. Hence, achievement of high affinity antibodies that can compete with other treatments such as antibiotics is of great importance in clinical setting. The use of rAbs for therapeutic purposes has become popular in recent years because these antibodies can overcome many of the

disadvantages of conventional immunotherapy methods. In a clinical trial, systemic administration of llama-derived nanobodies by intravenous or subcutaneous injections to more than 700 humans produced no adverse side effects (273). Moreover, local administration of rAbs to the GI tract, e.g. by oral or rectal administration, has been proposed. In this case, encapsulation of rAbs can be useful to protect them from damage caused by low gastric pH and pancreatic proteases (274). Furthermore, rectal administration, e.g. as a supplement to fecal microbiome transplantation (FMT), may also be possible (253, 275–277). For this purpose, immobilization of rAbs on beads may help their absorption and eliminate soluble toxins through the rectum.

Another advantage of using rAbs is that they can be used in the form of bispecific or multi-specific antibodies. Bispecific antibodies often maintain the properties of their parental antibodies, but they are more effective. Additionally, production of bispecific or multi-specific antibodies is of low cost and is preferred over combination therapies. In fact, the number of antibodies required to be developed is reduced through production of bispecific or multi-specific antibodies. Interestingly, a limitation of rAbs is their short half-life compared to mAbs, which can be increased by simple strategies such as genetic fusion to an albumin-specific nanobody and genetic fusion to the Fc domain of a conventional IgG antibody (278, 279). These strategies can protect rAbs from intracellular degradation (49, 129, 280, 281), and as a result, these methods are useful in prolonging the life of rAb fragments. However, bispecific or multi-specific rAbs may not need this reinforcement as their size confers sufficient *in vivo* half-life. In case of a bispecific tetramer designed based on an anti-toxin A and B, it was shown that this tetramer protects against death in CDI mice (217). This result confirmed the effectivity of these bispecific antibodies in reducing the severity of CDI and supports the hypothesis that increases in the size of sdAbs by using fusion constructs can improve the neutralization potency of sdAbs. Another major consideration is that the integration of protein engineering and rAbs technologies has led to the development of antibodies resistant to stomach acid and GI proteases. The use of different strategies, e.g. masking protease active sites, enzyme inhibition, pH modulation, and encapsulation, can improve the stability of antibodies (108). Additionally, the use of site-directed mutagenesis and genetic engineering can help select rAbs with high thermostability (282). In this regard, the use of disulfide engineering on anti-TcdB sdAbs enhanced thermostability and resistance to acidic pH without reducing their neutralization capabilities (214).

Expression of rAbs on the cell surface or as secretory proteins of *lactobaccili* is another feasible option (283, 284). Displaying rAbs fragments on probiotic bacterial surface is a two-way solution for disease control that helps in maintaining the gut microbiota and can preserve antibodies in the GI tract for a long time (283, 285–287). There are many advantages for this method, including cost-effective production, ease of administration, long shelf life, use of probiotic bacteria as complementary to treatment, and facility of genetic manipulation of bacteria, which make it an interesting topic for many researchers. This method has shown promising results in several GI infections (218). For instance, the use of anti-CDT sdAbs displayed on *lactobacilli* was capable to protect a hamster model of CDI (218). Interestingly, it was suggested that probiotic bacteria like *B. longum* have a much higher efficiency in secreting sdAbs in a functional form than scFvs (216).

Another key point is the possibility of using the sequence of these antibodies as an option in gene therapy. Gene therapy based on bispecific sdAb fragments against TcdA and TcdB could effectively neutralize toxins in animal models of CDI (217), and it is speculated to receive more attention in future research. Additionally, the application of scRNA-seq technologies for constructing or screening display libraries can help select antibodies with functional properties and high affinity (154, 155). These technologies can efficiently preserve the cognate V_H–V_L pairing and *in vivo* maturation of antibodies, thus will be further considered in the field of antibody therapy in the near future (159, 160). Overall, the effectiveness of rAbs can easily be enhanced by various methods and they can be employed as alternative therapeutics in future (260). We expect therefore to achieve practical information on desirable properties, efficacy and clinical applications of rAbs in the coming years.

Conclusion

Taken together, both mAb and rAb fragments (i.e., scFv and sdAb) are capable of CDI immunotherapy. However, a better understanding of *C. difficile* biology and the role of its virulence factors would help targeted treatment of severe CDI or rCDI caused by hypervirulent strains. Previous studies have demonstrated that rAbs, especially scFvs and sdAbs, have low molecular weight, high antigen affinity, good stability, and fast tissue penetration, which lead to their extremely wide applications in diagnosis, treatment, and prevention of diseases. As a result, these antibodies can be a reliable option for therapeutic purposes. In our opinion, the future trends and upcoming research on the development of specific antibodies for CDI treatment will focus on application of high-specificity biomolecules such as rAbs, especially the use of probiotic bacteria expressing rAbs, which can also maintain and improve the diversity and integrity of the gut microbiome. Additionally, integration of antibody therapy with FMT may augment the gut microbiota normalization of recipients and increase the efficiency of fecal transplant. Further investigations are needed to meet these claims and provide important

information regarding the application of rAbs-based treatments for clinicians and patients with CDI.

Author contributions

HR was involved in writing of the original draft and constructing tables. MA and AN-R were involved in constructing figures and conceptualization. AY was involved in preparing the draft of the manuscript, reviewing, and editing. HAA and MRZ were involved in reviewing and revising the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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