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# Rapid-format recombinant antibody-based methods for the diagnosis of *Clostridioides difficile* infection: Recent advances and perspectives

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*Clostridioides difficile*, the most common cause of nosocomial diarrhea, has been continuously reported as a worldwide problem in healthcare settings. Additionally, the emergence of hypervirulent strains of *C. difficile* has always been a critical concern and led to continuous efforts to develop more accurate diagnostic methods for detection of this recalcitrant pathogen. Currently, the diagnosis of *C. difficile* infection (CDI) is based on clinical manifestations and laboratory tests for detecting the bacterium and/or its toxins, which exhibit varied sensitivity and specificity. In this regard, development of rapid diagnostic techniques based on antibodies has demonstrated promising results in both research and clinical environments. Recently, application of recombinant antibody (rAb) technologies like phage display has provided a faster and more cost-effective approach for antibody production. The application of rAbs for developing ultrasensitive diagnostic tools ranging from immunoassays to immunosensors, has allowed the researchers to introduce new platforms with high sensitivity and specificity. Additionally, DNA encoding antibodies are directly accessible in these approaches, which enables the application of antibody engineering to increase their sensitivity and specificity. Here, we review the latest studies about the antibody-based ultrasensitive diagnostic platforms for detection of *C. difficile* bacteria, with an emphasis on rAb technologies.

## KEYWORDS

*Clostridioides difficile*, recombinant antibody, phage display, immunoassays, immunosensors

## Introduction

*Clostridioides difficile*, an anaerobic Gram-positive spore-forming bacillus, is a medically important pathogen and known as the main cause of diarrhea in humans globally (Burke and Lamont, 2014). *C. difficile* can asymptotically be present in the gut of healthy individuals (Furuya-Kanamori et al., 2015) or lead to infections with a wide spectrum of clinical disorders, including abdominal pain, diarrhea, pseudomembrane colitis (PMC), or even in some cases death (Burke and Lamont, 2014; Orrell and Melnyk, 2021). Currently, *C. difficile* infection (CDI) is identified as the major cause of nosocomial diseases associated with antibiotic therapy (in particular cephalosporins, clindamycin, metronidazole, and vancomycin) and healthcare-associated diarrhea in adults (Viswanathan et al., 2010; Cornely et al., 2012; Azimirad et al., 2020a, 2022). Additionally, other risk factors are involved in CDI incidence, including immunosuppression, previous hospitalization, age above 65 years, and the use of proton pump inhibitors (Surawicz et al., 2013; Eze et al., 2017; Azimirad et al., 2021). Notably, the spore-forming nature of *C. difficile* can be paired with its ability to rapidly colonize the intestine of patients and arises a critical challenge in infection control and treatment in both the community and healthcare settings (Paredes-Sabja et al., 2014; Castro-Córdova et al., 2021). In the last two decades, the number of CDI patients has been increasing (Depestele and Aronoff, 2013; Azimirad et al., 2020b; Baghani et al., 2020), so that in the USA, *C. difficile* imposes more than 453,000 illnesses per year, leading to 29,600 deaths. These estimates confirm that *C. difficile* is a major continuous burden to public health (Lessa et al., 2015; Kelly et al., 2021). On the other hand, CDI treatment contributed to substantial healthcare cost, and in the USA alone the cost associated with CDI management exceed \$4.8 billion annually (Dubberke and Olsen, 2012; Balsells et al., 2016). Additionally, antibiotic therapy for CDI can result in

further disruption of the normal gut microbiome and the development of hypervirulent strains of *C. difficile*. Alternatively, 20–30% of the patients with primary infection symptoms experience recurrent disease within 2–6 weeks after the completion of antibiotic treatment (Cornely et al., 2012; Raeisi et al., 2022a), which would be higher after secondary and tertiary CDI and more refractory to treatment regimens (Cornely et al., 2012; Eze et al., 2017). Therefore, rapid and accurate diagnosis of CDI has been a troublesome issue for disease management, implementation of infection control measures, and epidemiological monitoring (Burnham and Carroll, 2013; Peng et al., 2018). Additionally, an early and efficient diagnosis can positively impact the clinical outcome of CDI patients with primary infection and reduce the rate of infection recurrence (Barbut et al., 2014).

In recent years, antibodies, i.e., polyclonal (pAb) and monoclonal antibodies (mAbs), have been extensively applied for diagnostic and therapeutic purposes of various human diseases and cancers (Roovers et al., 2011; Ascoli and Aggeler, 2018; Haji-Hashemi et al., 2018; Raeisi, 2018; Raeisi et al., 2020; Zahavi and Weiner, 2020; Chiari et al., 2021; Hwang et al., 2022). In this regard, antibodies are exploited due to their high affinity and specific binding activity toward target molecules, which highlights their effectiveness as theranostics tools. In recent decades, recombinant antibody (rAb) technologies have widely received much attention as diagnostic tools (Xu et al., 2017; Shali et al., 2018; Fouladi et al., 2019; Raeisi et al., 2019; Alibeiki et al., 2020; Raeisi et al., 2020, 2022b; Berry et al., 2022). The rAb technologies that involve the construction of large libraries of different antibody fragments, such as fragment antigen-binding (Fab), single-chain fragment variable (scFv), minibodies, and nanobodies, can screen antibodies *in vitro* based on their binding properties, thus help develop more cost-effective antibodies with high sensitivity and specificity (Bockstaele et al., 2000; Angela Chiew Wen et al., 2016; Raeisi et al., 2022). This review will focus on currently available procedures for CDI diagnosis and different antibody-based rapid detection methods with an emphasis on rAbs. Additionally, analytical aspects of rAbs as recognition elements to develop ultrasensitive methods will be discussed.

## Conventional techniques for *Clostridioides difficile* detection

Some of the major and common *C. difficile* antigens are its surface proteins, including surface-layer proteins (SLPs), cell wall protein 66 (Cwp66) and 84 (CWP84), flagellin FliC, flagellar cap protein FliD, which play key role in attachment to intestinal mucus and have been used as target biomolecules in previous studies (Merrigan et al., 2013; Kandalaft et al., 2015; Shirvan and Aitken, 2016). Additionally, glutamate dehydrogenase (GDH) is a constitutive enzyme produced in large amounts by all toxigenic and non-toxigenic strains of *C. difficile* and can be easily detected in stool samples (Arimoto et al., 2016; Kelly et al., 2021). However,

Abbreviations: AP, Alkaline phosphatase; AAD, Antibiotic-associated diarrhea; CCNA, Cell-culture cytotoxicity neutralization assay; CLIA, Chemiluminescent immunoassay; Cwps, Cell wall protein; *C. difficile*, *Clostridioides difficile*; CDI, *C. difficile* infection; CROPs, Combined repetitive oligopeptide structures; CV, Cyclic voltammetry; EIA, Enzyme immunoassay; ELISA, Enzyme-linked immunosorbent assay; EMA, European Medicines Agency; FliC, Flagellin; FliD, Flagellin filament cap protein; Fabs, Fragment antigen binding domains; FDA, Food and Drug Administration; GI tract, Gastrointestinal tract; GTD, Glucosyltransferase domain; AuNPs, Gold nanoparticles; LFIs, Lateral flow immunoassays; LOD, Limit of detection; mAbs, Monoclonal antibodies; NAATs, Nucleic acid amplification tests; PCR, Polymerase chain reaction; pAbs, Polyclonal antibodies; PU, Polyurethane; PMC, Pseudomembranous colitis; QD, Quantum dots; QDNBs, Quantum dot nanobeads; QCM, Quartz crystal microbalance; rAbs, Recombinant antibodies; SARS-CoV-2, Severe acute respiratory syndrome coronavirus 2; SLP, S-layer proteins; scFv, Single-chain fragment variable; sdAb, Single-domain antibodies; SPR, Surface plasmon resonance; TC, Toxigenic culture; VH, Variable regions of heavy domain; VL, Variable regions of light domain; ZIKV, Zika virus.

the introduction of toxin-producing strains of *C. difficile*, as the main cause of antibiotic-associated diarrhea (AAD) in the 1970s, led to the application of several diagnostic methods to detect CDI, most of which relying on the detection of toxin A (TcdA) or B (TcdB) (Kelly et al., 2021). In fact, the main symptoms of CDI are contributed to the secretion of bacterial toxins (Di Bella et al., 2016) that affect the epithelial cells of the gastrointestinal (GI) tract by inactivating Rho/Ras proteins and subsequently lead to loss of epithelial barrier function, cytoskeleton disintegration, condensation of actin, severe inflammation, and eventually cell death (Chen et al., 2015a; Di Bella et al., 2016). These outcomes damage the patient's colonic mucosa and cause severe diarrhea and PMC. Some strains also express an additional toxin, named binary toxin CDT, but its role in the CDI pathogenesis has not been fully understood yet (Chandrasekaran and Lacy, 2017). Notably, the majority of the studies have demonstrated that only toxigenic strains, producing TcdA and/or TcdB, are pathogenic and can cause CDI (Di Bella et al., 2016; Chandrasekaran and Lacy, 2017). Moreover, based on the criteria declared by the European Society of Clinical Microbiology and Infectious Diseases (ESCMID), a CDI case is diagnosed when there are clinical symptoms compatible with CDI (usually diarrhea), stool test positive for TcdA and/or TcdB of *C. difficile* without evidence of another cause of diarrhea, and colonoscopy or histopathological data revealing PMC (van Prehn et al., 2021).

Currently, various methods have been introduced for detecting *C. difficile*, the most important of which being toxigenic culture (TC), cell-culture cytotoxicity neutralization assay (CCNA), enzyme immunoassay (EIA) for TcdA and/or TcdB, GDH detection, polymerase chain reaction (PCR), and stool culturing (Kelly et al., 2021). However, these diagnostic methods have several limitations in terms of speed, sensitivity, specificity, cost and ease-of-use (Gite et al., 2018). Generally, TC and CCNA are considered as reference methods for *C. difficile* identification by detecting toxin A and/or B (Planche and Wilcox, 2011). The application of the TC assay to stool samples leads to the isolation of *C. difficile* strains and determination of their ability to produce toxins. Although this test has shown high sensitivity (95%), it is very cumbersome, expensive, and time-consuming as it takes 3–5 days to be completed (Yücesoy et al., 2002). In addition, TC assays alone may show false-positive results due to the presence of non-toxigenic strains (Alcalá et al., 2008). On other hand, CCNAs have also shown high sensitivity and specificity (90–95%) for detecting toxigenic *C. difficile* strains, but these tests examine the production of toxins *in vitro*, which may not reveal correlations between fecal toxin levels and disease severity. In fact, *in vitro* examination may not reflect the actual *in vivo* toxin levels (Pollock, 2015).

The limitations of using cytotoxicity assays have led to the introduction of alternative techniques, including the application of numerous commercial qualitative EIA tests for detecting toxins A or/and B. These techniques have high specificity (96–98%) compared to toxigenic culture, but low sensitivity (52–75%) (Eastwood et al., 2009; Moon et al., 2016). Therefore, nucleic acid

detection techniques that are highly sensitive, should be used as a complementary method (Burnham and Carroll, 2013). However, nucleic acid amplification tests (NAATs) alone are not recommended because these tests can lead to false-positive results as they detect toxin-encoding genes of *C. difficile* regardless of toxin production (Gateau et al., 2018). Recently, detection of pathogen-specific proteins like GDH by EIA or latex particles has been proposed in some studies (Davies et al., 2015; Yoldas et al., 2016). GDH is easily detectable in stool samples and also known as a good screening marker for *C. difficile*. GDH detection methods are of high sensitivity but low specificity as GDH is highly abundant in both toxigenic and non-toxigenic strains, as a result, these methods are incapable to differentiate CDI from asymptomatic colonization or the presence of non-toxigenic strains (Surawicz et al., 2013), thus, additional EIA for detection of toxins A, B, or both is required (Badger et al., 2012; Kelly et al., 2021). A summary of the specificity and sensitivity of various diagnostic tests for CDI compared to toxigenic culture are presented in Table 1.

Presently, there is no agreement on the most appropriate laboratory tests used for CDI diagnosis but much clinical reliance is still on the detection of TcdA and/or TcdB by enzyme-linked immunosorbent assay (ELISA) platforms (Moon et al., 2016; Yoldas et al., 2016; Paitan et al., 2017). However, it is still controversial whether to give priority to detecting bacterial toxins or bacterial infection. Several studies have shown a significant correlation between the level of toxins in the stool and disease severity, as a result, determining the toxin concentrations in the stool can be helpful in disease management and predicting treatment outcomes (Planche et al., 2013; Di Bella et al., 2016; Pollock et al., 2019). Importantly, when the prevalence of *C. difficile* infection is low, no diagnostic test should be used alone because of low positive predictive values. Besides, dissimilarity of the results obtained from different diagnostic tests has made it difficult to consent to a single and reliable standard method, thus, the application of a simple and rapid technique with high sensitivity and specificity for *C. difficile* detection is challenging yet (Burnham et al., 2015; Song et al., 2015). Altogether, the diagnosis of CDI is often confirmed based on the positive results of two or three detection tests (Oldfield et al., 2014), and it is generally recommended to use the three-step algorithm as shown in Figure 1 for accurate CDI diagnosis.

In the past two decades, more rapid methods for detection of toxins of *C. difficile* have been developed and some of them are highly rapid and sensitive but are not yet commercially available (Jarrige et al., 2011; Pollock, 2015; Wilson et al., 2015; Gite et al., 2018). However, providing desirable performance characteristics such as being rapid, easy-to-use, and cost-effective can lead to clinical application of these emerging methods. So far, two commercial clinical biosensor kits have been introduced for CDI diagnosis that can detect the infection in a very short time (Table 2). Additionally, a wide variety of antibody-based diagnostic methods are available for detecting infections of the GI tract. Some of these methods, such as immunofluorescence or ELISA, are laboratory techniques and need trained personnel,

TABLE 1 Summary of diagnostic tests for *Clostridioides difficile* infection.

Test	Substance detected	Sensitivity %	Specificity %	Time required	Limitations	Reference
TC	<i>C. difficile</i>	95	80–90	3–5 days	Long turnaround time, trained personnel	Davies et al. (2009), Moon et al. (2016)
CCNA	Toxin	95	90–95	1–3 days	Long turnaround time, trained personnel, technical demands	Huang et al. (2009), Reller et al. (2010)
GDH	<i>C. difficile</i>	95–100	88–92	Hours	Low specificity, false-negative results	Eastwood et al. (2009), Cheng et al. (2015), Ramos et al. (2020)
EIA toxin	Toxin	51–80	98–99	Hours	Low sensitivity	Davies et al. (2009), Eastwood et al. (2009)
NAATs	<i>C. difficile</i>	92–97	83–100	Hours	Low specificity, costly and technical demands	Davies et al. (2009), Eastwood et al. (2009), Paitan et al. (2017)
GDH and EIA toxin	Toxigenic <i>C. difficile</i>	83–100	97–100	Hours	Dependent on toxin results, some variation in reported sensitivity	Kim et al. (2014), Cheng et al. (2015)
NAATs and EIA toxin	Toxigenic <i>C. difficile</i>	77–100	91–100	Hours	Some variation in reported sensitivity, technical demands	Huang et al. (2009), Humphries et al. (2013)

TC, toxigenic culture; CCNA, cell-culture cytotoxicity neutralization assay; EIA, enzyme immunoassay; GDH, glutamate dehydrogenase; NAATs, nucleic acid amplification tests.

while others, such as agglutination techniques, membrane EIAs, and lateral flow immunoassays (LFIA), are very simple and fast (Balsalobre-Arenas and Alarcón-Cavero, 2017). Different formats of antibodies applied in diagnostic tools are discussed below.

## Applicable formats of antibody for detection purposes

Antibody-based detection methods, like EIA, ELISA, and LFIA or immune-chromatographic tests (LFIA/ICT), are mostly designed based on the use of mAbs and pAbs in their platforms. The antibodies were first produced as pAbs and their production is still ongoing for various purposes. Normally, pAbs are made through the vaccination of animals such as rabbits, goats, and sheep, and can be rapidly generated at less expense and not requiring complicated technical skills (Stills, 2012). These molecules are produced by different B cell clones in the body, thus are usually a heterogeneous mixture, which can recognize multiple epitopes of antigen (Figure 2A). This property of pAbs can lead to the risk of low specificity and cross-reactivity with different targets (Ascoli and Aggeler, 2018).

The technique of mAb production, known as hybridoma technology, was invented by Kohler and Milstein in 1975 (Köhler and Milstein, 1975; Figure 2B). Although the hybridoma technology has been successful, it has many shortcomings, e.g., limited number of candidates, time-consuming, inefficiency to generate antibodies toward highly conserved antigens, self-antigens, sensitive antigens, and toxic antigens. A strategy that overcome the disadvantages of hybridoma is techniques based on *in vitro* antibody production, e.g., yeast display, ribosome display,

phage display, and mammalian cell display methods (Raeisi et al., 2022a; Valldorf et al., 2022), which among them, phage display and ribosome display are preferred in most laboratories due to their large diversity in the range of  $10^{12}$ – $10^{15}$  variants with high transformation efficiency (Kunamneni et al., 2020; Raeisi et al., 2022a). Here, we discuss the rAb techniques that are currently being studied for diagnosis proposes.

## *In vitro* recombinant antibody technologies

*In vitro* antibody display technologies, also called recombinant antibody technology, are prepared based on the diverse antibody genes contained in a specified library. The first display technology introduced was phage display, which was presented by Georg P. Smith in 1985 (Willats, 2003). Since these technologies depend on *in vitro* screening rounds, the production of antibodies could be much easier and more cost-effective than *in vivo* conditions (Alfaleh et al., 2020). A unique property of rAbs is the high affinity and specificity of them for detecting target molecules. Alternatively, the main concern in the development of immunoassays has always been the increase in the sensitivity and affinity of antibodies, which can be achieved by the possibility of integration of *in vitro* antibody display technologies with genetic engineering (83). In addition, using *in vitro* expression systems for the production of rAb fragments can finally lead to achieving sufficient amounts of antibodies for diagnostic purposes (Wang et al., 2013; Jensen et al., 2018) and allowing the construct of new different types of rAb fragments, including Fab, scFv, domain antibodies (dAbs), bivalent antibodies, multivalent antibodies,

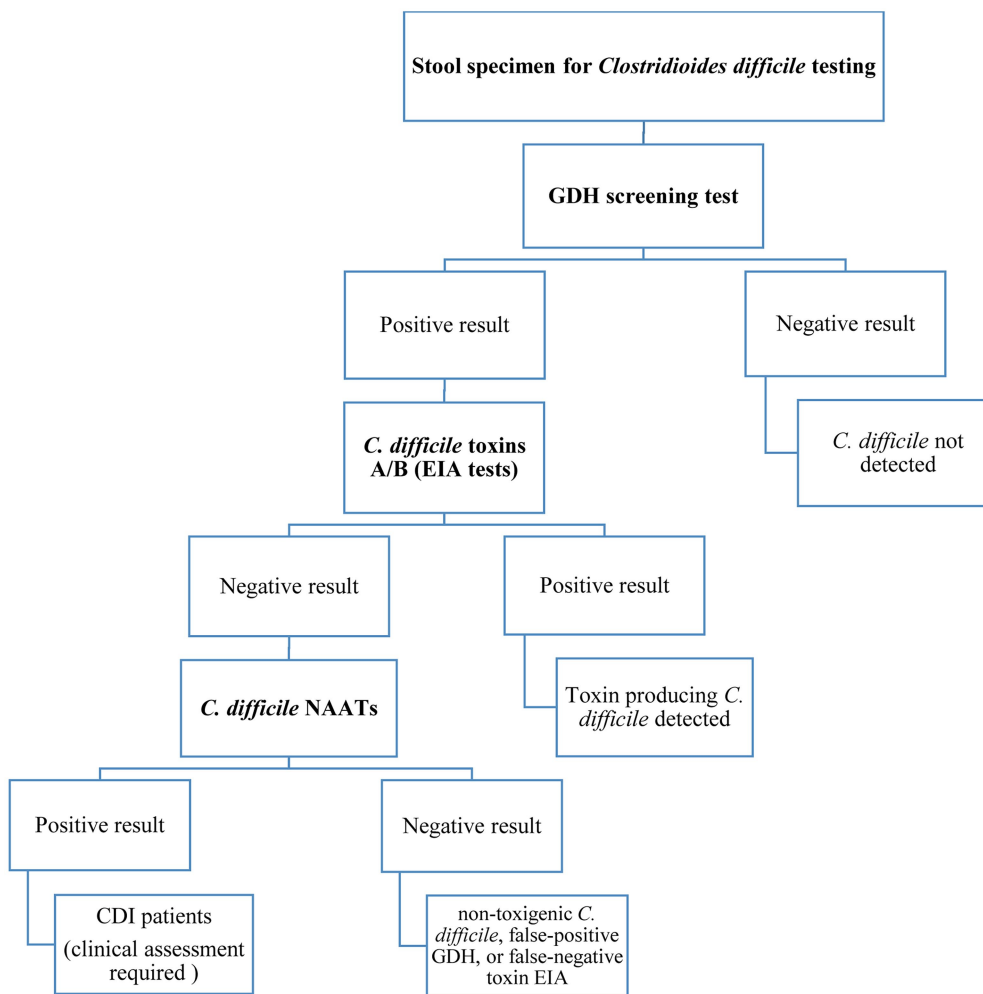


FIGURE 1  
Three-step algorithm for diagnosis of *Clostridioides difficile* infection.

TABLE 2 Examples of commercially available clinical biosensors kits for *Clostridioides difficile* infection.

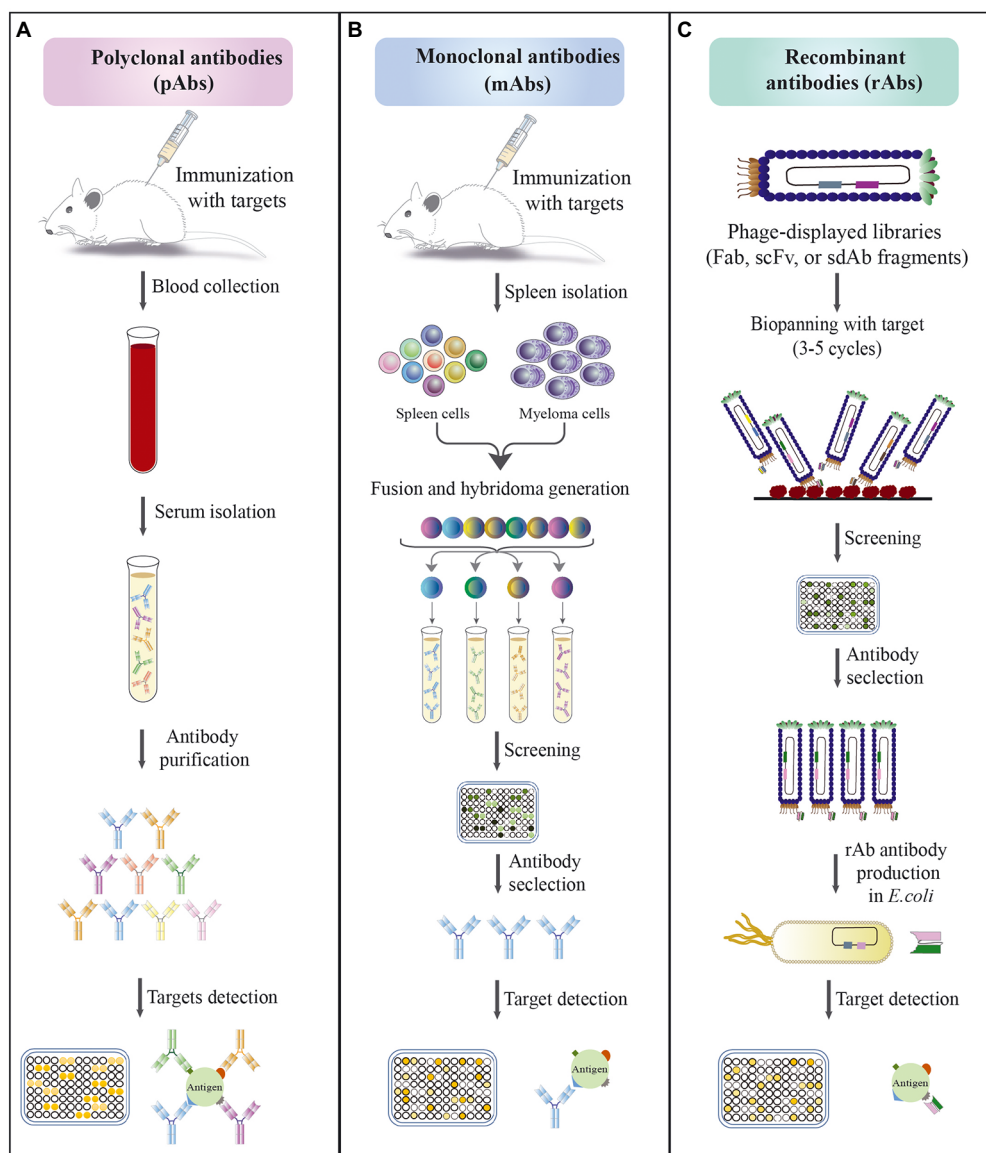
Platform	Target (analyte)	Transducer Platform Biosensor	Detection format	Applicability	LOD	Response time	Reference
Commercially available clinical biosensors	GDH ( <i>Clostridioides K-SeT</i> )	Membrane-based lateral flow assay	Optical	Stool	0.5 ng/mL	15 min	<a href="https://www.corisbio.com/products/Clostridioides-k-set">https://www.corisbio.com/products/Clostridioides-k-set</a>
	Whole cells (DR1107A, Oxoid, Hampshire, UK).	Rapid latex agglutination	Colorimetric latex agglutination	Selective media	N/A	2 min	<a href="https://www.thermofisher.com/order/catalog/product/DR1107A#/DR1107A">https://www.thermofisher.com/order/catalog/product/DR1107A#/DR1107A</a>

GDH, glutamate dehydrogenase; N/A, not available.

and bispecific antibodies (Yang et al., 2014; Fan et al., 2015; Høydahl et al., 2016). In terms of production of rAbs, ribosome display and phage display technologies have been introduced as potent *in vitro*, cell-free systems for the screening of large libraries, which can select high-affinity binders against various antigens without compromising the library (Li et al., 2019; Hammerling et al., 2020; Valldorf et al., 2022).

## Ribosome display and phage display technologies

Ribosome display technology is known as a powerful tool for the rapid isolation and direct evolution of high-affinity binders, particularly antibodies. This technology generates a stable complex containing antibody–ribosome–mRNA that can link



**FIGURE 2**  
Schematic approaches for the development of different types of antibodies. **(A)** Polyclonal antibodies production; the methods' steps include: (1) immunization of animals with an antigen to trigger immune response; (2) blood collection from immunized animals; (3) serum isolation; (4) antibody purification; (5) evaluation of the affinity of antibodies against antigen by immunoassay. **(B)** Monoclonal antibody production by hybridoma technique; the methods' steps include: (1) immunization of animals with an antigen to trigger immune response; (2) isolation of antibody-producing cells from the mouse spleen; (3) fusion of B cells with myeloma cells to produce hybridoma cells; (4) screening and selection of high affinity antibody produced by hybridoma cells; (5) evaluation of the specificity of selected antibodies against antigen by immunoassay. **(C)** Recombinant antibody production by phage display technology; the methods' steps include: (1) amplification of library; (2) exposure of the library to a surface coated with specific antigen; (3) elution of bound phages and their application for a new selection round; (4) screening of positive phage clones by ELISA after 3–5 rounds of biopanning; (5) selection of specific phages and their expression; (6) evaluation of the specificity of selected antibodies against antigen by immunoassay.

individual antibody fragments to their respective mRNA (Kunamneni et al., 2020). Conceptually, the ribosome display technique includes several steps, including library preparation, *in vitro* transcription and translation, screening and selecting a specific mRNA, and reverse transcription-PCR (RT-PCR) for further screening rounds or analysis (Li et al., 2019). Briefly, in this method, the mRNA lacks a termination codon (nonstop mRNA) at the end of the coding sequence, which holds the translating

ribosome at the end of mRNA and creates an unreleased nascent polypeptide. This process results in the formation of antibody–ribosome–mRNA complexes, allowing simultaneous isolation of antibody fragments and their corresponding mRNA through an affinity for an immobilized antigen. To isolate high-affinity protein–mRNA complexes, 3–5 rounds of panning are carried out, and selected complexes are subjected to *in situ* RT-PCR to recover the DNA encoding protein sequence (Kunamneni et al., 2020).

Within the past two decades, the production of rAbs, particularly scFvs, has been strikingly accelerated through ribosome display technology. Moreover, this technology has become a popular tool in medicine for basic research, disease diagnostics, and therapy (Park, 2020; Valldorf et al., 2022). However, one of the limitations of this method is the accessible, functional ribosome levels in the reaction for the library, which relates to the library size (Kunamneni et al., 2020). The ribosome display technology has been applied for selecting scFvs with high affinity against different targets such as tumor cells (Huang et al., 2018), Zika virus (ZIKV) (Kunamneni et al., 2018), *Mycobacterium tuberculosis* (Ahangarzadeh et al., 2017), and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (Chen et al., 2021). Moreover, it has been successfully used for scaffold selection, including affibodies (Lagoutte et al., 2019), however, there are no reports about antibody selection through ribosome display for CDI diagnosis or treatment. Overall, this method could be undoubtedly considered to select specific antibodies for new targets for diagnostic or therapeutic purposes in the future.

Additionally, the phage display technique is one of the most widely used technologies for the production of rAbs. In this technology, specific peptides or proteins are displayed on the surface of the filamentous phage particles through fusion between the genes encoding the antibody with the coat protein (pIII or pVIII) of the phage (Figure 2C). Therefore, a foreign phenotype is displayed on the phage surface that its genotype is retrievable in the phage (Schirrmann et al., 2011). The phage display technique contains the following steps: cloning of antibody-gene library, packaging of the resulting phagemids into phage particles presenting the respective antibody fragment on its surface, bio-panning to enrich antibodies binding to target structure, amplification of binding antibody phage, screening of individual antibody clones for the desired characteristics, e.g., by ELISA, subcloning of selected antibodies, and expression in format and expression system of choice (Hammers and Stanley, 2014). The most common method of bio-panning is coating antigens on a solid surface with high protein-binding capacity, such as polystyrene tubes (Jensen et al., 2018; Lu et al., 2020). In the next step, the surface coated by antigen is exposed to phage library, then, stringent washing of the surface is performed to remove nonbinding antibody phage. Subsequently, elution of the bound antibody phage is conducted by methods like

pH shift or by using trypsin, and then, reamplification is done by infection of *Escherichia coli*. Reamplified phage will be used in the next round of panning. It is expected that after each round of panning, the number of specific binding phages is increased. After 3–5 panning rounds, antigen-specific antibody phage will be checked by several methods such as ELISA, immunoblotting, or flow cytometry (Bazan et al., 2012). This approach is similarly done for the production of both therapeutic and diagnostic antibodies (Schirrmann et al., 2011; Frenzel et al., 2017).

The phage display technique has been widely used for detecting various targets, such as haptens, toxins, foreign and self-antigens (Tullila and Nevanen, 2017; Hussack et al., 2018; Raeisi et al., 2022a). For example, a specific antibody against a tumor marker expressed by breast tumor, named ErBb2 protein, has been selected through naïve antibody library that is useful in immunoassay tools (Bakir et al., 1993). Furthermore, many rAbs derived from phage libraries have been examined for diagnosis or therapy of bacterial diseases; the majority of these rAbs have been developed to facilitate disease detection and estimate the presence of contamination in environmental and food samples or levels of sample contamination (Kuhn et al., 2016; Priyanka et al., 2016). These rAbs are usually designed for various purposes, including detection of whole bacterial cells or toxins, blocking receptors, and modulating the host immune system (Kuhn et al., 2016), among them, whole bacterial cells are mostly applied for diagnostics (Byrne et al., 2013). An overview of rAbs derived from phage display libraries against *C. difficile* is presented in Table 3.

## Development of rapid detection methods based on recombinant antibodies against *Clostridioides difficile*

In recent decades, the design of rapid diagnostic methods based on pAb or mAb antibodies have been attracted much attention. Accordingly, a wide variety of antibody-based diagnostic methods are available for the diagnosis of GI tract infections, such as immunofluorescence, ELISA, and various immunosensors. Additionally, the rAb technologies are considered as powerful tools to provide next-generation immunoassays (Sang et al., 2015;

TABLE 3 Recombinant antibodies derived from phage display libraries against *Clostridioides difficile*.

Target	Antibody format	Antibody origin	Application	References
TcdB	scFv	Human	ELISA	Deng et al. (2003)
FliC, FliD	scFv	Human	ELISA, WB, <i>in vitro</i> assay	Shirvan and Aitken (2016)
SLP	sdAb	Llama	ELISA, WB, <i>in vitro</i> assay	Kandalaf et al. (2015)
TcdA	sdAb	Llama	ELISA, WB, <i>in vitro</i> neutralization	Hussack et al. (2011)
TcdA, TcdB	sdAb, bispecific	Alpaca	ELISA, <i>in vitro</i> assay, <i>in vivo</i> protection	Yang et al. (2014)
TcdB	scFv-Fc	Human	<i>in vitro</i> neutralization	Chung et al. (2018)
Binary CDT toxin	sdAb, sdAb-Fc	Llama	ELISA, IF	Unger et al. (2015)

TcdA, toxin A; TcdB, toxin B; SLP, surface-layer protein; scFv, single-chain fragment variable; sdAb, single-domain antibodies; Fc, fragment crystallizable region; ELISA, enzyme linked immunosorbent assays; WB, western blot; IF, immuno fluorescence microscopy.

Dinarelli et al., 2016). The advantages of these antibodies, e.g., ease of production in *E. coli* and its reproducibility, have made them much popular than conventional antibodies (Frenzel et al., 2017; Sun et al., 2018). More importantly, this single-chain format of antibodies can be easily fused with other proteins, such as detector enzymes, e.g., alkaline phosphatase (AP), resulting in formation of antibodies with multiple functions which can increase both the reaction rate and the sensitivity of diagnostic assays. In fact, the design of ELISA-based scFv-AP fusion proteins helps simultaneous use of two properties, namely binding specificity of antibody and AP enzyme activity. For instance, application of scFv-AP fusion proteins for different targets such as ractopamine (Dong et al., 2012), aflatoxin (Rangnoi et al., 2011), and glycocholic acid (Cui et al., 2018) have been described.

The following are examples of new diagnostic methods developed for detection of *C. difficile*. Moreover, various other diagnostic platforms based on rAb technology are well discussed.

## Enzyme-linked immunosorbent assay

One of the most favorite immunoassay techniques is ELISA. Due to its high performance, low cost, high speed and possibility to test a large number of samples simultaneously, ELISA-based kits became the standard technique for diagnosis of various agents (Mollarasouli et al., 2019). Developing toxin ELISAs as a rapid test that is based on mAbs or pAbs has always attracted much attention. For example, development of double-antibody sandwich (ds) ELISA by using mAb targeting the receptor binding region of TcdB (anti-CDB3) produced by hybridoma technologies was reported by Chen et al. which was regarded as an effective method for reliable diagnosis of CDI by detecting TcdB in diarrhea stools (Chen et al., 2015b).

Additionally, rAbs can be used as an alternative attractive strategy to mAbs and pAbs. Successful application of scFv antibodies in various ELISA platforms is reported in many studies; for instance, the development of ELISA based on phage-displayed scFv antibodies was reported for detecting *B. melitensis* (Hayhurst et al., 2003), IFN- $\gamma$  antigen (Yang et al., 2018), cowpea chlorotic dwarf virus (Raeisi et al., 2020), citrus tristeza virus (Raeisi et al., 2022b; Chen et al., 2015b), the protein components of type three secretion system of *Xanthomons citri* (Raeisi et al., 2019), *B. thuringiensis* (Dong et al., 2018) and so on. Moreover, several publications have reported the development of diagnostic VHH-based ELISA assays for rapid diagnosis of specific targets. Detection of influenza H5N1 virus (Zhu et al., 2014a), *B. thuringiensis* (Wang et al., 2014), *Listeria monocytogenes* (Tu et al., 2015), and ochratoxin A (Zhang et al., 2019) are few examples of VHHs application in ELISA platforms.

The application of rAbs for the quantitative detection of toxins can also be a reliable approach, which costs less than conventional ELISAs. There are some reports in the literature for successful application of rAbs in detection of toxins. In this regard, scFv antibodies against toxin B of *C. difficile* were reported by Deng

et al. (2003). The performance of anti-TcdB scFv fragments was shown by a sandwich ELISA and the results demonstrated that the isolated scFvs had high specificity to detect toxin B and did not have cross-reactivity with toxin B-negative *C. difficile* bacteria. Interestingly, isolated scFv fragment had higher sensitivity than the mAb and could detect a minimum of 10 ng of toxin B/well. In another study, Unger et al. (2015) evaluated the ability of anti-binary toxin sdAb fragments in blocking cytotoxic effect of the binary toxin, and results confirmed the proper function of sdAbs against this toxin; it was concluded that these sdAbs are promising new diagnostic tools for diseases associated with *C. difficile* (Unger et al., 2015).

## Chemiluminescent immunoassay

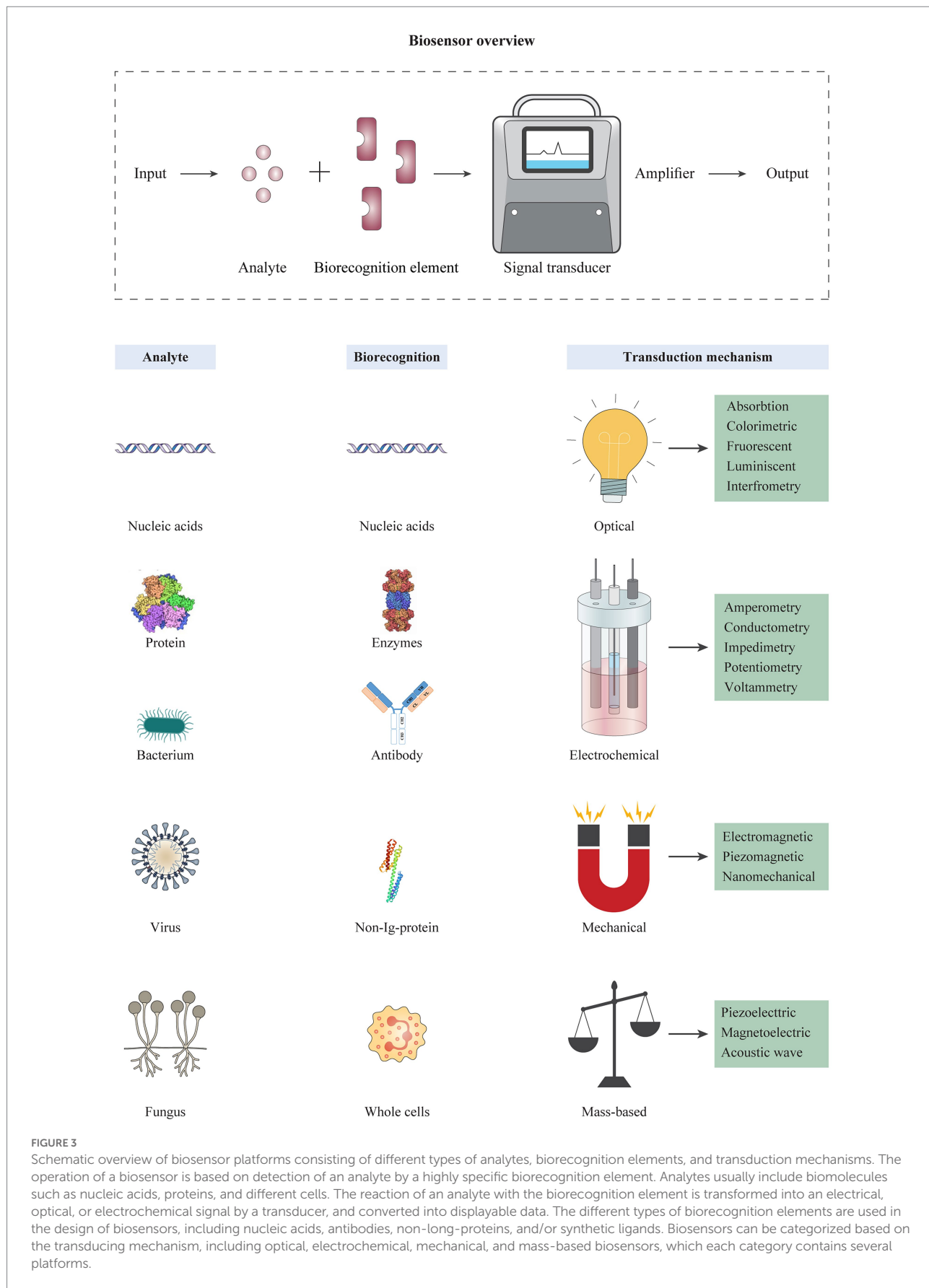
Chemiluminescent immunoassay (CLIA) is an immunoassay technique using luminescent molecule as an indicator of the analytic reaction that composes of combination of chemiluminescence techniques and immunochemical reactions (Cinquanta et al., 2017). This method is similar to other labeled immunoassay such as ELISA, except that CLIA substrates generate light emission in the presence of an enzyme, causing a more sensitive process compared to ELISA. Since CLIA technique has many advantages such as high sensitivity and specificity, rapidity, high stability of reagents, and compatibility with immunology assay protocols, it has been used to measure hormones, tumor markers, autoantibodies, and infectious disease markers (Chen et al., 2012).

Recently, use of magnetic-particle-based chemiluminescent enzyme immunoassay (CLEIA) for detection of *C. difficile* has been reported by Qi et al. (2020a). In this study, a CLEIA was developed using an anti-toxin B mAb-coated magnetic particles and anti-toxin B mAb conjugated to AP enzyme. The proposed CLEIA demonstrated high sensitivity for toxin B detection and showed a linear working range from 0.12 to 150 ng/mL with a limit of detection (LOD) of 0.47 ng/mL, which was similar to those of a commercial ELISA kit. This method could detect antigen in stool samples in less than 30 min, indicating high potential of the proposed method for rapid TcdB detection.

## Immunosensors

Antibodies can be used in the design of biosensors for rapid diagnosis purposes. Biosensors are known as devices for rapid detection or determination of the concentration of biological analytes like biomolecules, biological structures or microorganisms (Mehrotra, 2016). A biosensor typically consists of a biorecognition element and a transducer (Figure 3). A biorecognition element must identify a specific analyte and interact with it. The interaction between biorecognition element and analyte changes the biosensor's properties; these changes are converted by the transducer into a measurable signal that is





a form of energy, e.g., optical, thermal, and electrical. Thus, the encounter of sensors with specific pathogens or pathogenic byproducts leads to the production of positive detection signals (Bhalla et al., 2016). Classification of biosensors is based on the type of biorecognition element (e.g., enzyme, antibody, and nucleic acid probe) or the transducing method used (e.g., piezoelectric, optical, and electrochemical; Alhadrami, 2017), and different techniques are classified like nano- or micro-technology platforms accordingly. Importantly, integration of nanotechnology with biosensor systems can augment the analytical efficiency of detection methods and provide more sensitive and accurate diagnosis (Zhu et al., 2014b; Pan et al., 2017; Cho et al., 2020). This technique helps to improve characteristics like sensitivity (e.g., low LODs), assaying time, and analytical procedures. In this regard, gold nanoparticles (AuNPs) are known as one of the most widely used nanomaterials in biomedical research and clinical imaging (Hammami et al., 2021). Biocompatibility, non-toxicity, ease of characterization, and high stability are some of the significant properties of AuNPs that promote their diverse applications (Cabuzu et al., 2015; Hu et al., 2020). So far, there are successful reports for the use of these compounds to detect various viruses and bacterial agents (Mocan et al., 2017; Singh et al., 2017).

Antibody-based sensors or immunosensors, are a popular format of biosensors that change the transducer signal when a specific antigen–antibody reaction is detected (Cho et al., 2020). Various immunosensors have been developed and they have gained much attention in biochemical analyses (Tang and Xia, 2008), clinical diagnoses (Morris, 2013), food quality control (Scognamiglio et al., 2014), and environmental monitoring (Mehrotra, 2016). Therefore, they seem to be excellent candidates for rapid and sensitive diagnosis of diseases and pathogens as far as there are many reports of their use for detection of bacteria such as *E. coli* (Park et al., 2008), *Salmonella enterica* (Cinti et al., 2017), *Salmonella typhimurium* (Fulgione et al., 2018), and *X. citri* (Haji-Hashemi et al., 2018). Immunosensors can be designed on the basis of different types of antibodies (pAbs, mAbs, and rAbs), however, most of the immunosensors are designed based on pAbs and mAbs.

Additionally, fragment antibodies such as sdAbs, scFvs, and Fabs have emerged as suitable alternatives to design biosensors (Ahmad et al., 2012). Although rAbs have several significant advantages over conventional antibodies, they are yet not fully exploited in fabrication of immunosensors. There are some reports for application of rAbs in designing various immunosensors, e.g., optical biosensors (Damborský et al., 2016; Peltomaa et al., 2018), electrochemical detection (Grewal et al., 2013), LFIA (McDonnell et al., 2010; Kim et al., 2020), and piezo-immunosensors (Shen et al., 2007). However, most sensors designed to detect *C. difficile* are based on nucleic acid probes, aptamers or mAbs, examples of which are given in Table 4, there are limited research published on biosensors that are designed based on rAbs. Below, application of different immunosensors for rapid detection of *C. difficile* are discussed.

## Electrochemical immunoassays

Among different types of biosensors, electrochemical immunoassays have gained much attention as a bioanalytical method. These diagnostic methods can become a promising approach with high sensitivity and specificity in the future; they have many advantages like ease of signal quantification, low cost, rapidity, high compatibility, high repeatability, high sensitivity, ease of integration, and miniaturization (Kimmel et al., 2012; Cho et al., 2020). Design of a mAb-based electrochemical immunoassay is a successful example of application of electrochemical immunosensor for detecting *C. difficile* (Fang et al., 2013). In particular, Fang and co-workers developed a simple sandwich-assay type electrochemical immunosensor to detect TcdB by using graphene oxide (GO) as a scaffold to improve the surface area to capture a large number of primary antibodies. In this system, horseradish peroxidase (HRP)-labeled secondary TcdB antibody was also introduced on the electrode surface through the unique properties of AuNPs. Coating antibodies with nanoparticles caused a large amount of antibody entering the biosensor system leading to an increase in electrochemical impedance signal, and as a result the sensitivity in diagnosis was increased, so that this system showed a linear working range from 3 pg/mL to 320 ng/mL with a LOD of 0.5 pg/mL under optimal experimental conditions, which was much lower than that of other conventional methods like ELISA. More importantly, high specificity of the produced electrochemical immunosensor for detecting TcdB in stool samples, indicated that application of this immunosensor can be a promising option in clinical diagnosis of CDI.

Application of AuNPs coated by specific antibodies to detect *C. difficile* or its toxins has been also reported by Zhu et al. (2014b). In this work, an electrochemical impedance immunosensor based on AuNPs coated with anti-toxin sdAb antibodies for detecting both TcdA and TcdB was designed (Zhu et al., 2014b). The results showed LODs of 0.61 and 0.60 pg/mL for TcdA and TcdB, respectively, that are indicative of high sensitivity of this method. Evaluation of immunosensor efficiency for detecting toxins in stool samples showed that the designed biosensor can be potentially used in clinical applications. These promising results prove that the use of AuNPs in immunosensors led to an excellent performance with high sensitivity. However, this method requires a relatively complex procedure that is not easily available.

In another study, Cui et al. (2018) developed a label-free electrochemical biosensor for TcdB detection. In this work, sdAb isolated against TcdB was used for preparing polyurethane (PU) electrodes with nanopiked gold electrode-based label-free electrochemical immunosensor. Signal amplification in this method was six times as high as flat PU gold electrode-based immunosensor. Based on these results, the PU nanopiked gold electrode-based immunosensor can be an excellent option for rapid detection of TcdB especially when low cost and simple processing is expected (Cui et al., 2018). Recently, the use of silica

TABLE 4 Examples of rapid detection used for *Clostridioides difficile* infection.

Biorecognition elements	Target (analyte)	Platform	Detection format	Applicability	LOD	Response time	References
Nucleic acid	Spores	Magnetic bead aggregation	LAMP coupled to PiBA	Selective media	N/A	N/A	DuVall et al. (2016)
	Toxins	Magnetic	NMR-based technology	Stool	<180 CFU/mL	45 min	Yang et al. (2017)
	TcdA/B	Magnetic	ELISA sandwich assays	Stool	1.35 $\mu$ g/mL	N/A	Hong et al. (2015)
	TcdA/B	MEF	Optical	Stool	10 CFU/mL	40 s	Joshi et al. (2014)
	TcdB	PEPS		Electrical impedance (PEPS) (direct detection)	Stool	150 CFU/mL	40 min
Aptamer (aptasensor)	TcdA/B	FMSM	Optical	Stool	1.12 ng/mL	20 min	Yang et al. (2020)
	TcdA/B, binary toxin	Magnetic Beads/SOMAmer	ELISA sandwich assays	Selective media	1 pg/mL to 300 pg/mL	N/A	Ochsner et al. (2013), Ochsner et al. (2014)
	GDH	Fluorescence emission structure-switching fluorescence signaling aptamer	Optical	Stool	1 pg/mL	60 min	Liu et al. (2018)
	TcdA	Colorimetric	Electrochemical impedance	Stool	1 pg/mL	N/A	Luo et al. (2014)
	TcdB	Colorimetric	Colorimetric assay	Stool	600 and 60 pg/mL	30 min	Liu et al. (2020)
	TcdB	Colorimetric	Colorimetric assay	Stool	5 ng/mL	30 min 60 min	Luo and Liu (2020)
	GDH	Colorimetric	Colorimetric assay	Selective media	10 $\mu$ g/mL	30 min	Hui et al. (2018)
Enzyme based	Specific protease, PPEP-1	Bioluminescent Sensor	Optical	Stool	10 $\mu$ g/mL	30 min	Ng et al. (2021)
Antibody	TcdB	Amperometric	Electrochemical	TcdB	0.3 ng/mL	30 min	Xu et al. (2014)
	TcdA/B	Impedance	Electrochemical	Stool	0.6 pg/mL	30 min	Zhu et al. (2014b)
	TcdB	DPV	Electrochemical	Stool	0.7 pg/mL	45 min	Fang et al. (2013)
	GDH	Lateral flow immunoassay	Optical, thermal	Selective media	1.0625 ng/mL 0.1328 ng/ mL	N/A	Wang et al. (2016)
	TcdB	Magnetic and fluorescent particles	Automated ELISAs	Stool	45 pg/mL	30 min	Gite et al. (2018)
	Toxins	Single-molecule array technology	Automated ELISAs	Stool	45 pg/mL	N/A	Banz et al. (2018)
	TcdB	Impedance polyurethane (PU) nanospiked gold electrode	Electrochemical (DPV)	Stool	0.5 pg/mL	40 min	Cui et al. (2018)
	TcdA/B	Fluorescence	Optical	Stool	N/A	40 s	Joshi et al. (2014)
	SlpA, TcdB	SERS-based LFA	Optical	Stool	0.01 pg/mL	20 min	Hassanain et al. (2021)
	GDH, TcdB	Split-luciferase assay, luminescent	Optical	Stool	4.5 pg/mL 2 pg/mL	32 min	Adamson et al. (2022)

GDH, glutamate dehydrogenase; TcdA, toxin A; TcdB, toxin B; LAMP, microfluidic loop-mediated isothermal amplification; PiBA, product-inhibited bead aggregation; MEF, metal enhanced fluorescence; MAMEF, microwave accelerated metalenhanced fluorescence; PEPS, piezoelectric plate sensor; FMSM, fluorescent magnetic spore-based microrobot; ELISAs, enzyme-linked immunosorbent assays; PPEP-1, pro-pro endopeptidase 1; DPV, differential pulse voltammetry; SERS-based LFA, surface enhanced Raman scattering-based lateral flow assay; N/A, not available.

nanoparticles in designing electrochemical biosensor has led to sensitive and specific detection; this technique was able to sensitively detect bacteria in five minutes by cyclic voltammetry measurements, and interestingly, this device could detect other microorganisms with minor modifications within its features (Mathelie-Guinlet et al., 2019), and can be considered as a potential diagnostic platform in future.

## Optical biosensors

In addition to electrochemical immunosensor, optical biosensors are a popular form of detection methods and known as a powerful alternative to conventional analytical techniques, particularly for their small size, high sensitivity, and cost effectiveness (Damborský et al., 2016). Diagnosis made by optical biosensors is based on sensitive detection of photon emission from dyes and molecules excited by light. Hence, fluorescent probes are the most widely used probes in these biosensors that can emit photons after interacting with specific targets such as antigens, antibodies, or genomic material. The fluorescence emission helps to increase the sensitivity; thus, the results obtained by these methods are more reliable than conventional detection methods such as microscopy or enzyme-based detection. These biosensors are widely reported for detecting bacteria, such as *S. typhimurium* (Seo et al., 1999), *E. coli* (Riangrunroj et al., 2019), and *S. enterica* (Quintela et al., 2019).

Generally, optical biosensors have been applied in different platforms. In this regard, the application of MultiPath immunoassay technology that uses non-magnified digital imaging to count molecular targets labeled microscopic fluorescent particles has been reported by Gite et al. (2018). In this work, a novel MultiPath immunoassay technology was developed for highly sensitive detection of *C. difficile* toxin B antigen based on anti-TcdB mAbs, which are conjugated to magnetic and fluorescent particles through a carboxyl linkage (EDC/NHS chemistry; Gite et al., 2018). This technology counts and images target-specific magnetic and fluorescent particles banded together by anti-TcdB in minimally processed stool samples. This technology can efficiently detect CDI due to its advantages including minimal sample preparation, rapid diagnosis, and requiring simple optical equipment. This study demonstrates that the designed immunosensor has an equivalent performance to cytotoxicity assay in terms of sensitivity for toxin B detection, so that the results showed LOD of 45 pg/mL with 97% sensitivity; 98.3% specificity; and 98.2% accuracy for the MultiPath TcdB test, which was similar to cytotoxicity assays and significantly better than on-market EIAs. In addition, this method shows superiority to NAATs because it directly detects the toxins secreted by vegetative *C. difficile* cells even at very low concentrations, while the NAATs detects the toxin in dormant spores which do not cause disease, thus, false positive results can be avoided. Recently, the use of single-molecule array (Simoa) technology was described for ultrasensitive detection of toxins (Song et al., 2015). Simoa technology, also known as digital ELISA, is based on efficient capture, labeling and detection of single protein molecules on

paramagnetic beads in arrays of femtoliter-sized wells. This method has a very low LOD, and it is about 1,000-fold more sensitive than conventional ELISA (Rissin et al., 2010). There is no report on the use of specific anti-*C. difficile* rAbs using this technology and only the design of Simoa technology based on mAbs was reported for CDI diagnosis. The development of Simoa technology for detecting TcdA and TcdB by Song et al. (2015) showed that this method can detect all strains producing toxins in the stool specimens with LODs of 0.45 and 1.50 pg/mL for TcdA and TcdB, respectively (Song et al., 2015). In another work, Sandlund et al. (2018) introduced an automated single-molecule counting technology for detecting *C. difficile* toxin A and B (Sandlund et al., 2018) in stool samples with a low LOD of 2.0 pg/mL for TcdA and 0.7 pg/mL for TcdB, which was similar to Simoa technology; however, the dependence of both methods on fluorescent labeling complicates and limits their operations and use.

## Lateral flow immunoassays

Recently, researchers have made undeniable efforts to develop assays that do not rely on laboratory equipment. LFIA as one of such examples is a rapid technique which has attracted a lot of attention. Today, LFIA is widely used for various monitoring and diagnostic purposes, particularly for on-site use in veterinary to serve as analytical tests for a range of biochemical analytes, for medical and food safety purposes, home-pregnancy test (Koczula and Gallotta, n.d.), human immunodeficiency virus (HIV) diagnosis (Nguyen et al., 2009), and SARS-CoV-2 detection (Moshe et al., 2021). The assay is performed on a sheet-shaped matrix, e.g., cellulose, containing freely labeled antibodies with a color or the fluorescent mark. The assay implements where an analyte interacts with a detection reagent and an immobilized capture reagent. Several types of antibodies (mAb, pAb, rAb, HRP-conjugated, AP-conjugated, etc.) are applied in LFIAs. Notably, LFIAs are known as a suitable approach to implement on-site and they are also compatible with already available commercial tests. Due to their customer-friendly and low-cost features, they would be potentially applied in diagnostic platforms in future (Liu et al., 2021). Application of these devices has many advantages as they are fast, easy to use, portable, and they do not require external equipment (Koczula and Gallotta, 2016), leading to the popularity of these biosensors among researchers. Currently, application of LFIA methods for determination of harmful substances in food is being investigated. The use of LFIAs to detect *Enterococcus faecalis*, *Staphylococcus aureus* (Wang et al., 2017a), *L. monocytogenes* (Wang et al., 2017b), *Shigella* (Wang et al., 2016a), and *Leptospirosis* (Nurul Najian et al., 2016) has been successful so far. Combination of LFIA methods with nanoparticles, including AuNPs (Sun et al., 2015; Fu et al., 2017; Jiang et al., 2017), fluorescent-microsphere-derived ICA (F-ICA), and time-resolved Eu/Tb (III) nanoparticles ICA (TRF-ICA) (Tang et al., 2017; Zhang et al., 2017) was

suggested in biomarkers designing. Furthermore, application of nanoparticles such as gold in LFIA tests causes a thermal contrast that leads to increased sensitivity of detection systems. In a recent study, the sensitivity of visual or colorimetric readers was improved up to 8-fold for *C. difficile* (reduced from 1.0625 to 0.1328 ng/mL), thus, thermal contrast readers would receive much attention in future (Wang et al., 2016b).

In addition to lateral-flow assays, latex agglutination techniques (LAT) allow for naked-eye detection. LAT assays are based on the use of latex particles bound to antibodies, so that the antigen-binding sites are exposed and they can bind the target antigen. The binding of antibody to antigen leads to a lattice formation of the latex particles which appears as a visible agglutination. These types of techniques are rapid, easy to perform, inexpensive, fast and they do not require special equipment. The use of particles which change in color when aggregated, e.g., AuNPs, makes the visual detection possible in these techniques. In this context, rapid visual detection of bacteria such as *M. tuberculosis* (Bhaskar et al., 2004) and *E. coli* (Huang et al., 2001) was documented. Integration of this technique with nanoparticles like AuNPs increases the speed of this method in a way that target antigens can be detected in less than 30 min (Koczula and Gallotta, 2016). There is only one report on the use of antibody-latex agglutination test in detection of *C. difficile*, in which positive latex results were confirmed by cytotoxicity assay. Accordingly, this test can be an acceptable diagnostic tool for screening *C. difficile* toxins (Shahrabadi et al., 1984), which can be considered a simple and affordable diagnostic tool for clinical use.

Recently, the results of research on quantum dots (QD) have revealed that they are one of the best candidates for development of novel detection methods (Matea et al., 2017). QDs are very small nanoparticles with strong fluorescence properties that act as semi-conductors. The use of QDs in the tools developed for diagnosis of different diseases has been described (Zhao and Zeng, 2015). These diagnostic methods are semi-quantitative or quantitative and have many advantages such as being simple, fast, and easy, and they require only visual inspection. Additionally, QD technology has advantages over organic chemical fluorescent groups, such as higher quantum yield, wide absorption cross-sections, excellent optical reliability, and adjustable specification of fluorescent transmission (Dubertret et al., 2002).

Use of QDs in *C. difficile* detection has been reported; Qi et al. (2020) developed a LFIA using an anti-TcdB mAb coupled to quantum dot nanobeads (QDNBs). The LOD of QDNBs-LFA in the fecal samples was 0.297 ng/mL that was consistent with those of a commercial ELISA kit. Moreover, concerning sensitivity, QDNBs-LFA showed good correlations with clinical diagnosis. Thus, the performance of QDNBs in this diagnostic system was highly applicable for designing portable and rapid on-the-spot platforms (Qi et al., 2020b). Overall, the use of rAbs can be suggested to increase the efficiency of these biosensors (Sharma et al., 2016; Lara and Perez-Potti, 2018), and should not be ignored in future studies.

## Discussion

The current status of CDI diagnosis is still challenging. The available laboratory tests have remained confined and revealed a high frequency of inconsistency in detecting *C. difficile* colonization or diagnosis of suspected CDI patients (McDonald et al., 2018). Additionally, an accurate and early diagnosis of CDI is essential for optimal patient care, timely infection control, pharmacological treatment, and preventing the spread of infection (Gerding et al., 2016; Gateau et al., 2018; Peng et al., 2018). Therefore, the achievement of timely and accurate diagnostic assays with high sensitivity and specificity will play an increasingly pivotal role in more efficient disease management. Today, the development of diagnostic tools based on antibodies has received great attention. Some properties of antibodies, such as their high affinity and specific binding to target molecules, make them reliable therapeutic/diagnostic tools (Ahmad et al., 2012; Sharma et al., 2016). This has led to the introduction of numerous antibody-based diagnostic kits so far, however, obtaining antibodies with specificity, sensitivity, and high affinity has always been a challenge facing researchers (Hammers and Stanley, 2014). Recently, some studies have focused on the application of antibodies for CDI diagnosis; and further research is still being conducted in this area (Fang et al., 2013; Davies et al., 2015; Qi et al., 2020). Most of the research has been done on the production of antibodies using the hybridoma technology, which has been applied successfully in numerous cases, while it has many shortcomings, such as being time-consuming and inefficient in generating antibodies toward highly-conserved antigens, self-antigens, sensitive, and toxic antigens (Parray et al., 2020). In these cases, it is recommended to use alternative methods like non-antibody binding proteins (or protein scaffolds) and *in vitro* antibody display technologies (Alfaleh et al., 2020; Gebauer and Skerra, 2020). In contrast to monoclonal antibodies, protein scaffolds are single polypeptide chain structures without disulfide bridges or post-translational modifications that show high solubility and stability and can allow the selection for affinity, stability, and enzymatic activity (Simeon and Chen, 2018; Gebauer and Skerra, 2020). Additionally, *in vitro* antibody display technologies have many advantages, including the ability to be genetically modified to increase selectivity, specificity, and sensitivity, ease of production in *E. coli*, and reproducibility (Frenzel et al., 2017; Chiu et al., 2019; Raeisi et al., 2022a). Hence, various studies have suggested the use of phage display-derived antibodies for diagnostic purposes (Chamorro and Merkoçi, 2016; Mohd Ali et al., 2021). Innovative developments based on antibodies (e.g. various biosensor platforms) can provide rapid, reliable, and sensitive detection capability, which help the accurate diagnosis of specific targets. Importantly, due to the increase in public health concerns about nosocomial CDI, designing immunosensors can be proposed for rapid detection and disease management (Zhu et al., 2014b). Additionally, the combination of nanotechnology and various biosensor platforms has attracted a lot of attention as an excellent approach for developing fast, highly,

sensitive and specific devices for the diagnosis of bacterial and viral infections (Prasad, 2014; Park et al., 2016), which can improve the quality and precision of disease diagnosis (Castillo et al., 2020; Younis et al., 2020).

Application of rAb fragments (scFv, sdAb, etc.) to develop immunosensors or immunoassays has several distinct advantages, the most important of them is that rAb fragments can be readily produced *in vitro*. In addition, rAbs are easily formatted through genetic engineering or chemical conjugation for coupling antibodies to the sensors used to detect antigens. These advantages in the case of antibodies that have limited *in vivo* production, especially anti-toxins, are more highlighted. Another advantage of rAb antibodies is that in scFv-based label-free immunosensors, for example, piezo-immunosensors, a high-affinity rAb is sufficient to detect the antigen, thus no antigen-specific secondary antibody (i.e., detecting) is required (Shen et al., 2005). In fact, the use of rAb in these biosensors simplifies antigen detection because in some cases, the generation of just one antigen-specific antibody is difficult, let alone two. Furthermore, chemical modification of a sensor's surface under controlled conditions can facilitate correct orientation (e.g., on AuNPs) of rAb fragments at high density, which leads to improved avidity and sensitivity of the assay (Gandhi et al., 2018). In many sensing transducers and imaging technologies, rAb antibodies can be used instead of traditional whole antibodies, including quartz crystal microbalance (QCM), cyclic voltammetry (CV), surface plasmon resonance (SPR), and many other detection techniques (Zeng et al., 2012). These techniques can provide a tool with high sensitivity and specificity to detect antigens in complex samples like feces and blood. Moreover, a QCM based on rAbs can readily sense a change in mass on the sensor's surface, so that antibody concentration on the immunosensor surface can be easily determined to ensure that the same concentration of antibody is used every time and this helps enhance inter-assay reproducibility. This feature is not available for most traditional immunoassays (e.g., ELISAs) (Zeng et al., 2012). Interestingly, reducing the distance between donors and acceptors in optical biosensors increases their efficiency (Damborský et al., 2016), thus the use of small-sized antibodies like rAbs can be considered as a way to increase the efficiency of these biosensors (Sharma et al., 2016; Lara and Perez-Potti, 2018). So far, the application of rAbs in various diagnostic platforms, such as ELISA, LFIA, nanoparticles, and microfluidics, has been reported (Goodchild et al., 2005; Zhang et al., 2012). It seems that due to the desirable properties of rAbs, they can be employed for designing newer diagnostic tools in the future.

## Conclusion

Presently, the precise and effective diagnosis of CDI is based on a multistep approach. Thus, the application of rapid and accurate diagnostic tools can be a key step in the management of CDI. Previous studies have demonstrated that rAbs possess high affinity and specificity for the detection of various targets and can be considered as reliable diagnostic tools in CDI management.

Moreover, *in vitro* antibody production can be cost-effective compared with the conventional antibodies. This is particularly remarkable for developing novel and rapid detection tests such as biosensors. The effectiveness of rAbs can be enhanced by genetic engineering that would allow designing high performance diagnostic techniques and reducing the assay costs. Therefore, antibody generation by rAb technologies will provide an attractive platform for current and future diagnostic purposes and can be the future trend of research for designing ultrasensitive methods for CDI diagnosis.

## Author contributions

HR was involved in literature review, writing of the original draft, and figures illustration. MA was involved in literature review. AY was involved in conceptualization, preparing the draft of the manuscript, reviewing, and editing. HAA and MRZ were involved in 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.

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