Preparation and preliminary application of monoclonal antibodies to the receptor binding region of *Clostridium difficile* toxin B

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Abstract. A previous nationwide Chinese epidemiological study revealed through isolation of A⁻B⁺ *Clostridium difficile* strains, which produce toxin B (TcdB), but not toxin A TcdA, that the strains are widespread and more frequent in east Asian countries,. The development of a process capable of detecting TcdB is required in microbiological laboratories in order to facilitate the control of the A⁻B⁺ *C. difficile* strains, however, no diagnostic reagents have been developed to date. The aim of the present study was to prepare monoclonal antibodies (mAbs) targeting the receptor binding region of TcdB (CDB3), and to establish a double-antibody sandwich enzyme-linked immunosorbent assay (ds-ELISA), which can be used for the diagnosis of *C. difficile* infection. The recombinant protein, glutathione S transferase (GST)-CDB3 was expressed and purified using an *Escherichia coli* system. BALB/c mice were immunized with GST-CDB3 recombinant protein. A hybridoma technique was used for the production of anti-CDB3 mAb. The hybridoma clones were then screened using indirect ELISA, and anti-CDB3 mAb was produced in the ascites of the BALB/c mice. Isotyping of anti-CDB3 mAb was performed using an SBA Clonotyping system/horseradish peroxidase (HRP) ELISA kit. Protein G affinity chromatography was used for purification of anti-CDB3 mAbs, and the titer and specificity of the anti-CDB3 mAbs were assessed using indirect ELISA and western blot analysis, respectively. The ds-ELISA was established using HRP-labeled anti-CDB3 mAbd, which were used to detect TcdB clinically in diarrhea stools. A total of five stable hybridoma cell clones (1E7B, 1F8D3, 2F8A6, 3B6F1 and 4A4G2) producing anti-CDB3 mAb were established. The results of the present study indicated that the immunoglobulin (Ig)G isotype was predominant, as 1E7B2 IgG1 (λ), 2F8A6 IgG2a (κ) and 4A4G2 IgG1 (κ). In addition, the highest titer of anti-CDB3 mAb (2F8A6 and 4A4G2) was 1:51,200. Western

blotting revealed that the 2F8A6 and 4A4G2 mAbs recognized the CDB3 protein specifically. Following anti-CDB3 mAb (4A4G2) HRP-labeling, the optimal working concentration was confirmed to be 1:400, and the concentration of coated antibody (2F8A6) was 20 µg/ml. The sensitivity of the ds-ELISA was 73.33% for the A⁺B⁺ toxigenic *C. difficile* strains, and 86.67% for the A⁻B⁺ toxigenic *C. difficile* strains, with a specificity of 100% for all. In conclusion, the present study successfully developed novel mAbs specific to CDB3, and developed a ds-ELISA kit with high specificity and sensitivity for the rapid detection of TcdB. This offers a useful tool for the diagnostic assessment of TcdB.

Introduction

Clostridium difficile was first detected in the fecal flora of healthy newborns in the mid-1930s (1) and considered to be non-pathogenic until identified as the primary cause of pseudomembranous colitis in 1978 (2). *C. difficile* is a gram-positive anaerobic, spore-forming bacterium, and accounts for 15-30% of all episodes of antibiotic-associated diarrhea (AAD) and 95-100% of cases of pseudomembranous colitis (PMC) (3,4). The morbidity, mortality and relapse rates of the diseases caused by *C. difficile* have markedly increased in hospital and community settings due to the spread of hypervirulent strains, including BI/NAP1/027, restriction endonuclease analysis type BI, North American pulsed-field gel electrophoresis type 1 and polymerase chain reaction ribotype 027 (5,6), and due to the improper administration of antibiotics in the past decade (7,8). Based on several reports from the US, Canada and Europe, the incidence of *C. difficile* infection (CDI) has increased between 2- and 4-fold in the past decade, particularly in elderly patients exposed to health care settings, including long-term care facilities and hospitals (9-11). Children and peripartum females, previously described as low risk populations for CDI, now show increased incidence (12). CDI-induced mortality rates have markedly increased between 3,000/year in 1999-2000 and 14,000/year in 2006-2007 (13). The risk of recurrence following an initial episode of CDI is 20%, which increases up to 60% following a third episode (14). CDI remains the most common cause of hospital-acquired diarrhea, with the number of hospitalized patients diagnosed with any CDI discharge increasing between 139,000 in 2000 and 336,600 in 2009, at a cost of $1,000,000,000 annually (15). In addition, CDI has surpassed methicillin-resistance *Staphylococcus aureus* as

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the most common hospital and healthcare facility-associated infection (16). *C. difficile* toxin A (TcdA), an enterotoxin, and *C. difficile* toxin B (TcdB), a cytotoxin, are the major virulence factors of *C. difficile,* and contribute to its pathogenicity by inducing mucosal inflammation and diarrhea (17). In addition to these toxins, *C. difficile* may produce a number of other putative virulence factors, including *C. difficile* binary toxin (CDT), fibronectin binding protein A, fimbriae, S-layer protein A, Cwp84 cysteine protease and Cwp66 and CwpV adhesions (17).

A previous nationwide epidemiological study demonstrated that isolation of an A^-B^+ *Clostridium (C.) difficile* strain, which produces TcdB, but not TcdA, has spread widely (18-20) and is more frequent in east Asian countries (8,19,21-23). Initially, TcdA was considered to be the most important factor responsible for diarrheal disease (20), however, several observations in variety of animal models have demonstrated this is not always the case. In hamsters and mice, intragastric administration of TcdA, but not TcdB, resulted in intestinal fluid accumulation, diarrhea, hemorrhage and mortality (24). However, previous reports have demonstrated that A^-B^+ *C. difficile* strains can lead to similar symptoms, ranging between mild diarrhea and severe pseudomembranous colitis (25,26). Diagnosis of CDI via the detection of TcdB is required in microbiological laboratories in order to control these strains of *C. difficile*, however, to date no such diagnostic reagents have been developed in China. Therefore, rapid diagnosis of *C. difficile* in patients with AAD and PMC is important for guiding the treatment and control of nosocomial infection spread. The primary objective of the present study was to establish hybridoma cell lines stably secreting anti-CDB3 monoclonal antibodies (mAbs), which may be used for the development of double-antibody sandwich (ds)-ELISA. In addition, the present study aimed to detect TcdB in diarrhea stools using ds-ELISA, in order to establish an effective method for the future diagnosis of CDI.

Materials and methods

Ethical statement. Six female BALB/c mice (age, 6-8 weeks old; weight, ~20 g) were purchased from the Laboratory Animal Center of the Central South University (Changsha, China) and housed under pathogen-free conditions at 18-28°C, 40-70% humidity with free access to standard chow and water. All experiments were performed according to the guidelines of the Animal Ethics Committee of the Central South University. The mice were sacrificed using rapid cervical dislocation to minimize animal suffering.

Expression of glutathione S transferase (GST)-CDB3 recombinant protein. The recombinant plasmid pGEX-4T-1-CDB3 of *Escherichia coli* BL21 (DE3) was constructed in the Clinical Laboratory in Xiangya Hospital of Central South University. It was transformed into an BL21(DE3) competent strain (American Type Culture Collection, Manassas, VA, USA), and the *E. coli* strains was induced. The optimal expression of recombinant proteins was achieved by regulating the concentration of bacteria prior to induction [optical density (OD), 600], along with the induction temperature, induction time and the concentration of isopropyl-β-D-thiogalactopyranoside (IPTG; Sigma-Aldrich, St. Louis, MO, USA), details of

which are shown in Table I. Following induction, the bacteria were harvested and centrifuged at 4°C for 15 min at 4,449 x g, and the pellet was resuspended in lysis buffer (Beijing Donglinchangsheng Biotechnology Co., Ltd., Beijing, China) containing 0.15 M NaCl, 0.05 M Tris-HCl, 0.1% SDS (Beijing Donglinchangsheng Biotechnology Co., Ltd.), 1% Triton X-100, 1% sodium deoxycholate (pH 7.2), followed by lysis by sonication in ice and centrifugation at 4°C for 10 min at 10,012 x g. *C. difficile* strains expressing the GST-CDB3 were also grown, as described above. The expression of the GST-CDB3 recombinant protein was analyzed using 10% SDS-PAGE. The SDS-PAGE gel was placed in Coomassie Brilliant Blue dye (Sangon Biotech, Co., Ltd., Shanghai, China) overnight in room temperature, then washed with distilled water three times.

Purification and identification of recombinant protein GST-CDB3. The GST-CDB3 recombinant protein was purified using a GST Sefinose™ kit (Sangon Biotech, Co., Ltd.) and dialyzed in 1X phosphate-buffered saline (PBS) at 4°C overnight, prior to being condensed to a high concentration. The recombinant protein concentration was determined using a Bicinchoninic Acid (BCA) Protein Assay kit (Thermo Fischer Scientific, Inc., Waltham, MA, USA) according to the manufacturer's instructions. The expression of the recombinant protein was analyzed using western blotting, as previously described (27). Briefly, the affinity purified recombinant GST-CDB3 protein was incubated at 72°C for 10 min in 1X NuPage SDS loading buffer (Beijing Donglinchangsheng Biotechnology Co., Ltd.), prior to being separated by 10% SDS-PAGE under native and denaturing conditions, and then transferred onto a nitrocellulose membrane (EMD Millipore, Billerica, MA, USA). Following blocking with 5% fat-free milk at 37°C for 2 h, the membrane was incubated with mouse anti-GST monoclonal primary antibody (1:2,000; P09211; EMD Millipore) at 37°C for 2 h, followed by incubation with polyclonal horseradish peroxidase (HRP)-conjugated goat anti-mouse secondary antibody (1:1,000; M8645; Sigma-Aldrich) at 37°C for 1 h. Between each incubation step, the membranes were washed five times with PBS and 0.2% Tween 20 (PBST; Beijing Donglinchangsheng Biotechnology Co., Ltd.). The bands were visualized using an enhanced chemiluminescence (ECL) system (Pierce Biotechnology, Inc., Rockford, IL, USA).

Immunization of mice. BALB/c mice were used for hybridoma production, as previously described (28). Briefly, 8-week-old female BALB/c mice were intraperitoneally injected three times with 50 μg purified GST-CDB3 recombinant protein at 2 week intervals. Complete and incomplete Freund's adjuvant (1:1; Sigma-Aldrich) was used to emulsify the protein for the first and subsequent immunizations. At 10 days following the third immunization, ELISA (Sangon Biotech, Co., Ltd.) was performed, as previously described (29), to confirm that the serum contained antibodies targeting recombinant protein GST-CDB3, and to detect the antibody titer of mouse serum (using the tail vein procedure). Recombinant protein GST-CDB3 was used as a coating antigen, 1% BSA-PBS as a blocking solution, mouse serum as the primary antibody, and HRP-conjugated goat anti-mouse immunoglobulin

Table I. L9(34) orthogonal test for the optimal expression of recombinant proteins.

Level	Concentration IPTG (mmol/l)	Induction time (h)	Concentration of bacteria (OD 600)	Induction temperature (˚C)
1	0.6	10	0.6	20
2	0.8	12	0.8	30
3	1.0	16	1.0	37

IPTG, isopropyl-β-D-thiogalactopyranoside; OD, optical density.

(Ig) G polyclonal antibodies (1:3,000; Sigma-Aldrich) as the secondary antibody. The mouse with the highest titer specific for GST-CDB3, determined using indirect ELISA, was then administered with 100 μg recombinant protein GST-CDB3 without adjuvant 1 week later. Subsequently, 3 days after the last immunization, the spleen was removed and macerated to obtain B lymphocytes, and then washed twice with RPMI-1640 medium (Gibco Life Technologies, Carlsbad, CA, USA).

Hybridoma production. Myeloma cells and spleen lymphocytes were prepared for fusion, as previously described (30). The SP2/0 mouse myeloma cell line (Cancer Research Laboratory, Central South University) in the logarithmic growth phase and with good morphological appearance was used as a fusion partner, the SP2/0 cells were cultured in 10% fetal bovine serum (FBS; GE Healthcare Life Sciences, Logan, UT USA) and RPMI-1640 medium at 37˚C in an atmosphere containing 5% CO_2, prior to treatment with 8-azaguanine (Sigma-Aldrich). The feeder cells of the peritoneal macrophages were collected from normal BALB/c mice by peritoneal washing with RPMI-1640 medium supplemented with hypoxanthine ($1x10^4$ M), aminopterin ($4x10^7$ M) and thymidine ($1.6x10^5$ M; Sigma-Aldrich) and seeded into 96-well plates 1 day prior to fusion. The splenic lymphocytes and SP2/0 cells were fused at a ratio of 5-10:1. This protocol involved the incubation of the cells in 1 ml 50% (w/w) polyethylene glycol/dimethylsulfoxide 3350 (Sigma-Aldrich) at 37˚C for 1 min, followed by washing with RPMI-1640 medium for 5 min. The hybridomas were seeded into 96-well plates containing the feeder cells and screened using hypoxanthine-aminopterin-thymidine medium. Growth of the hybridomas was regularly observed by an optical microscope (BIO500 PH; Olympus, Tokyo, Japan).

Screening of positive clones and cloning. At 2 weeks following fusion, the cell clones producing anti-CDB3 antibody were screened and identified by indirect ELISA, using the (0.89 mg/ml) GST-CDB3 recombinant protein and 1.21 mg/ml GST protein as coating antigens, 1% BSA-PBS as a blocking solution, the supernatant of each hybridoma cell clone as the primary antibody, and HRP-conjugated goat anti-mouse IgG polyclonal antibodies (1:3,000; Sigma-Aldrich) as the secondary antibody. The optical density (OD) was read at 450 nm using a microplate reader (Tianshi Technologies, Co., Ltd., Beijing, China and considered positive when the ratio of OD450 experimental to OD450 control was greater than three-fold. The wells, which were positive for the GST-CDB3 recombinant protein, but negative for GST protein were considered positive, and were sub-cloned using limiting dilution, as previously described (31). Further cloning was performed between three and five times in hypoxanthine-thymidine medium (Sigma-Aldrich) for 2 weeks until all selected hybridomas were positive. Finally, the positive clones in the 96-well plates were transferred into 24-well plates in RPMI-1640 medium supplemented without FBS. The strain of hybridoma cells, which established stable growth with the highest positive expression was selected, expanded and preserved at -70˚C.

Preparation and titer determination of anti-CDB3 mAb. The production of hybridomas from ascitic fluid was performed, as previously described by Harlow *et al* (31). The BALB/c mice were intraperitoneally injected with 0.5 ml incomplete Freund's adjuvant. After 7 days, the mice were intraperitoneally injected with $5x10^6$ hybridoma cells diluted in D-Hank's buffered solution (Sigma-Aldrich), which exhibited optimal results following indirect ELISA. The ascitic fluid was collected after 2 weeks and centrifuged for 5 min at 2,780 x g to remove cellular deposition and other sediments. The anti-CDB3 mAb supernatant was then diluted into a series of gradients (1:100, 1:200, 1:400, 1:800, 1:1,600, 1:3,200, 1:6,400, 1:12,800, 1:25,600, 1:51,200, 1:102,400 and 1:204,800), and indirect ELISA was performed using recombinant protein GST-CDB3 as a coating antigen, 1% BSA-PBS as a blocking solution, the serial dilutions of supernatant as the primary antibody, and HRP-conjugated goat anti-mouse IgG polyclonal antibodies (1:3,000; Sigma-Aldrich) as the secondary antibody, in order to detect the titer of anti-CDB3 mAb. The OD was read at 450 nm.

Isotype determination and purification of anti-CDB3 mAb. The antibody isotyping of anti-CDB3 mAb was performed using an SBA Clonotyping system/HRP ELISA kit (SouthernBiotech, Birmingham, AL, USA) containing goat anti-mouse IgG1, IgG2a, IgG2b, IgG3, IgA, IgM, Igκ and Igλ, according to the manufacturer's instructions. The anti-CDB3 IgG antibodies (5 ml) were added with an equivalen volume of precooled ammonium sulfate overnight in 4˚C, and then centrifuged at 4˚C for 15 min at 1,738 x g. The supernatant was decanted, and the precipitation was solubilized in PBS (0.02 M; 5 ml) and purified using Protein G affinity chromatography (GE Healthcare Life Sciences), according to the manufacturer's instructions. The positive components confirmed by indirect ELISA were dialyzed and condensed to a high concentration. Reduction was performed using

mercaptoethanol (Sigma-Aldrich) and separation was performed using 10% SDS-PAGE. Concentration was determined using a BCA Protein Assay kit.

Determination of affinity using ELISA. The present study determined affinity levels using ELISA, as previously described (32). Briefly, ELISA plates pre-coated with 5 μg/ml GST-CDB3 recombinant protein were blocked with 2% FBS at 37˚C for 2 h. Serial dilutions of the purified mAb were incubated at 37˚C for 2 h. The plates were then rinsed and incubated with HRP-conjugated goat anti-mouse IgG polyclonal antibodies (1:3,000) at 37˚C for 1 h. OD was measured at 450 nm and curves were constructed using the OD450 values obtained from the serial dilutions of the purified mAb, in order to determine the relative affinity.

Western blot analysis of anti-CDB3 mAb specificity. The GST-CDB3 recombinant protein was cleaved using thrombin (Sigma-Aldrich) and separated using 10% SDS-PAGE. The proteins were transferred onto a nitrocellulose membrane by electroblotting, prior to being blocked with 5% fat-free milk at 37˚C for 2 h. The membrane was incubated with purified anti-CDB3 mAb (1:1,000) overnight at 4˚C. Following three washes with PBST, the membrane was incubated with HRP-conjugated goat anti-mouse IgG polyclonal antibodies (1:1,000; Sigma-Aldrich) in 37˚C for 1 h. Following further washing the membrane was visualized using an ECL system.

Conjugation of mAb to HRP. The conjugation of mAb to HRP was performed, as previously described (33). A total of 2.6 mg HRP dissolved in 1.0 ml water and 0.1 ml sodium periodate solution (0.1 mol/l) were combined, stirred for 20 min, and dialyzed in 1 mM sodium acetate buffer (Sangon Biotech, Co., Ltd.; pH 4.4) overnight at 4˚C. A total of 0.5 ml (0.16 M) was subsequently added to the solution and stirred at 4˚C for 1 h. A total of 6 mg monoclonal 4A4G2 in 1 ml sodium carbonate buffer (0.01 M, pH 9.6) was added and stirred for 2 h. A total of 0.1 ml sodium borohydride solution (4 mg/ml) was added and dialyzed in 0.02 M PBS (pH 7.2) overnight at 4˚C, then precipitated by the addition of equal volumes of 100% ammonium sulfate (4˚C, 4 h), prior to centrifugation at 3,000 x g for 5 min, dialyzed against 0.01 M PBS (pH 7.4) overnight at 4˚C, and stored at 4˚C following dilution with 500 ml/l glycerol (1:1).

Establishment and optimization of ds-ELISA. A ds-ELISA for TcdB was established using 4A4G2 as a HRP-labeled mAb and 2F8A6 as a capture mAb. A chessboard titration method was designed to examine the optimal working concentration of capture antibody and HRP-labeled mAb. Briefly, a 96-well immunoplate was coated with purified anti-CDB3 mAb (2F8A6; concentrations, 20 μg/ml, 10 μg/ml, 5 μg/ml, 2.5 μg/ml, 1.25 μg/ml and 0.625 μg/ml) in 0.05 M/l carbonate buffer (pH 9.6) overnight at 4˚C. Following blocking with 200 μl 2% BSA-PBS overnight at 4˚C, the plate was washed five times in PBST, and 100 μl serial dilutions of GST-CDB3 recombinant protein in PBS were added to each well and incubated at 37˚C for 2 h, followed by a further five washes. At total of 100 μl diluted HRP-conjugate anti-CDB3 mAb (1:50, 1:100, 1:200, 1:400, 1:800, 1:1,600, 1:3,200, 1:6,400 and 1:12,800;

detecting antibody) was subsequently added, and the plates were incubated at 37˚C for 1 h. The plates were then washed and incubated at 37˚C for 30 min in the dark, following which then the OD450 was measured.

Stool sample collection. This study was conducted at, and approved by, the Xiangya Hospital of Central South University (Hunan, China) between June 2011 and November 2012. Patients with diarrhea were enrolled which was defined as more than three loose watery stool passages per day, on consecutive days or six times within 36 h. CDI was diagnosed when the *Clostridium difficile* was isolated from stool culture and identified by API20A (BioMerieux, Marcy l'Etoile, France), the *Clostridium difficile* isolates were also positive for toxin genes (tcdA, tcdB) as shown by multiplex PCR. The non-CDC group was defined as a group of patients who did not have diarrhea or had diarrhea but were negative for toxin A/B.

Analysis of clinical stool samples using ds-ELISA. A total of 45 diarrhea stool samples, including 15 samples containing A+B+ toxigenic *C. difficile*, 15 samples containing A+B+ toxigenic *C. difficile* and 15 samples negative for *C. difficile,* were determined using anaerobic toxigenic culture. Briefly, selective enrichment culture for *C. difficile* was performed, and the levels of TcdA, TcdB, CDTA, CDTB were determined using polymerase chain reaction (PCR). All experimental procedures and specimen collections were approved by the Ethics Committee of the Xiangya Hospital of Central South University. The expression levels of TcdB in the stool samples were analyzed using the ds-ELISA technique, as described above.

Fecal culture. Each stool sample (2 ml) was added to an equal volume of absolute ethanol, mixed and left at room temperature for 1 h, then centrifuged (4,449 x g/10 min) and the pellet was inoculated on selective *Clostridium difficile* Moxalactam-Norfloxacin-Taurocholate agar (CDMN-TA; Oxoid Ltd., Cambridge, UK) supplemented with 7% horse blood after alcohol shock (10), and incubated at 35±2˚C in an anaerobic atmosphere (85% nitrogen, 10% hydrogen and 5% carbon dioxide) for 48 h. Suspect *Clostridium difficile* colonies were subcultured and identified as *Clostridium difficile* on the basis of their morphology on agar plates and by Gram staining as well as the characteristic odor. In addition, oxygen resistance experiments were conducted on specific colonies and confirmed by API 20A (BioMerieux). The demonstration of toxin production was conducted by multiplex PCR for the toxin genes (tcdA and tcdB) or a positive toxin assay using the VIDAS® *Clostridium difficile* Toxin A & B (BioMerieux, France). Strains were stored at -80˚C in cryobank tubes until further testing.

Multiplex PCR. Toxin gene DNA was isolated from colonies of *Clostridium difficile* using a DNeasy blood and tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. All isolates were tested for the presence of tcdA, tcdB, cdtA and cdtB genes. The oligonucleotide primers used are shown in Table II. The amplification reactions were performed in 25 μl final volume containing 12.5 μl Taq PCR Master mix (2X; Beijing Tiangen Biotech Co., Ltd., Beijing,

Table II. Primer sequences of oligonucleotides was used for conventional PCR.

PCR product	Primer	Sequence (5'-3')	Fragment length (bp)
tcdA	NK1	5-GGACATGGTAAAGATGAATTC-3	546
	NK2	5-CCCAATAGAAGATTCAATATTAAGCTT-3	
tcdB	NK104	5-GTGTAGCAATGAAAGTCCAAGTTTACGC-3	204
	NK105	5-CACTTAGCTCTTTGATTGCTGCACCT-3	
cdtA	Cdta pos	5-TGAACCTGGAAAAGGTGATG-3	375
	Cdta rev	5-AGGATTATTTACTGGACCATTTG-3	
cdtB	Cdtb pos	5-CTTATTGCAAGTAAATACTGAG-3	510
	Cdtb rev	5-ACCGGATCTCTTGCTTCAGTC-3	

PCR, polymerase chain reaction.

Table III. Polymerase chain reaction conditions.

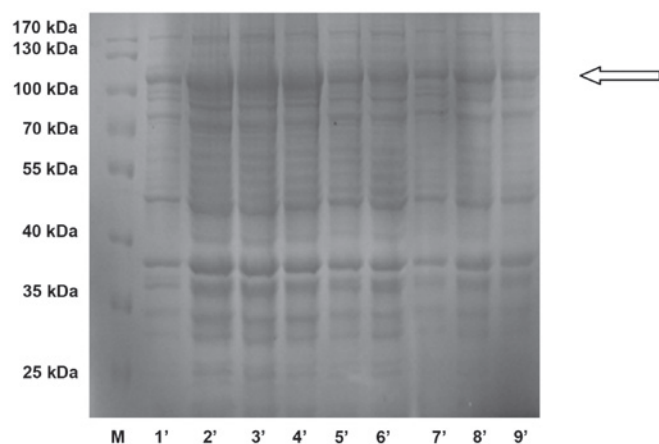| PCR product	Reaction conditions					
	Initial enzyme activation	Cycles	Denaturation	Annealing	Elongation	Final elongation
tcdA	95°C, 1 min	30	95°C, 15 sec	62°C, 2 min	72°C, 40 sec	72°C, 10 min
tcd B	95°C, 1 min	30	95°C, 20 sec	55°C, 30 sec	60°C, 2 min	72°C, 10 min
cdtA/cdtB	94°C, 1 min	30	94°C, 45 sec	52°C, 1 min	72°C, 80 sec	72°C, 10 min

Figure 1. Expression of purified recombinant GST-CDB3 protein in *Escherichia coli* was analyzed using 10% SDS-PAGE. The proteins were visualized by Coomassie Blue R-250 staining. Lane 1, protein molecular weight marker; Lane 2, purified GST-CDB3 recombinant protein; Lane 3, molecular size. M, protein molecular weight marker; 1', A1B1C1D1; 2', A1B2C2D2; 3', A1B3C3D3; 4', A2B1C2D3; 5', A2B2C3D1; 6', A2B3C1D2; 7', A3B1C3D2; 8', A3B2C1D3; and 9', A3B3C2D1. A was the concentration of IPTG (mmol/l); B was the induction time (h); C was the concentration of bacteria (OD 600); D was induction temperature (°C). 1,2,3 was the level of each, as shown in Table 1. GST, glutathione S transferase.

China), 1 μl each primer (10 μmol/l), 8.5 μl water and 2 μl DNA. The reaction conditions of PCR are shown in Table III. Gels were run under standard conditions on 1.0% agarose for 25 min at 100 V, using a 100-2,000 bp ladder (Fermentas, Pittsburgh. PA, USA) as size standard and stained with ethidium bromide for visualization.

Results

Expression of GST-CDB3 recombinant protein. The optimal conditions for inducing the expression of the GST-CDB3 recombinant protein, which was the optimal concentration of bacteria prior to induction, was 0.8 nM, and that of IPTG was 0.6 mM, determined using OD600. The optimal induction duration was 12 h and the induction temperature was 30°C. The GST-CDB3 recombinant protein was predominantly expressed as a soluble protein.

Purification and identification of GST-CDB3 recombinant protein. The GST-CDB3 recombinant protein was purified using a GST Sefinose™ kit under native conditions, identified using 10% SDS-PAGE and subsequently stained using Coomassie Brilliant Blue R250 (Fig. 1). The molecular weight of the GST-CDB3 recombinant protein was 96 kDa. The protein was then further identified using western blot analysis with anti-GST tag antibody (Fig. 2). The recombinant protein concentration was 0.89 mg/ml.

Preparation of anti-CDB3 mAb. In the present study, the rate of cell fusion was 98.4% (945/960), and a total of five (1E7B, 1F8D3, 2F8A6, 3B6F1 and 4A4G2) positive hybridoma cell lines stably secreting anti-CDB3 mAb were identified: 1E7B2 IgG1 (λ), 1F8D3 IgM (κ), 2F8A6 IgG2a (κ), 3B6F1 IgM (λ) and 4A4G2 IgG1 (κ). The titer was assayed using indirect ELISA, obtaining the following dilutions: 1E7B2 (1:25,600), 1F8D3 (1:12,800), 2F8A6 (1:51,200), 3B6F1 (1:25,600) and 4A4G2 (1:51,200). The 1E7B2, 2F8A6 and 4A4G2
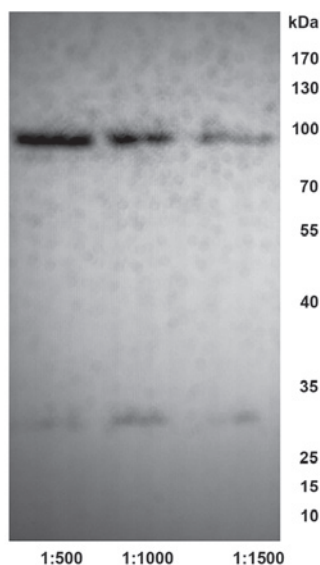
Figure 4. Relative affinities of purified anti-receptor binding region of *Clostridium difficile* toxin B mAbs were analyzed using an indirect ELISA assay of the serially diluted mAbs. mAbs, monoclonal antibodies. The relative affinities of the mAbs were defined by the antibody concentration at which the optical density value reached half the maximal signal at the plateau stage of antigen-antibody binding.

Figure 2. Western blot analysis of purified GST-CDB3 recombinant protein. Identification of proteins was confirmed by western blot analysis using anti-GST tag mAb (lane 1, 1:500; lane 2, 1:1,000; lane 3, 1:2,000). The band appeared most marked when the anti-GST tag mAb was diluted to 1:500. mAb, monoclonal antibody; GST, glutathione S transferase.

Figure 5. Western blot analysis of the purified 1E7B2, 2F8A6, 4A4G2 anti-receptor binding region of *Clostridium difficile* toxin B monoclonal antibodies. Western blotting results demonstrated that the 1E7B2, 2F8A6 and 4A4G2 anti-CDB3 mAbs reacted specifically to the large unit of the GST-CDB3 recombinant protein cleaved by thrombin (molecular weight, ~70 kDa) and the uncleaved GST-CDB3 recombinant protein (molecular weight, ~100 kDa), however, no reaction with the tagged protein GST was observed (molecular weight, ~26 kDa).
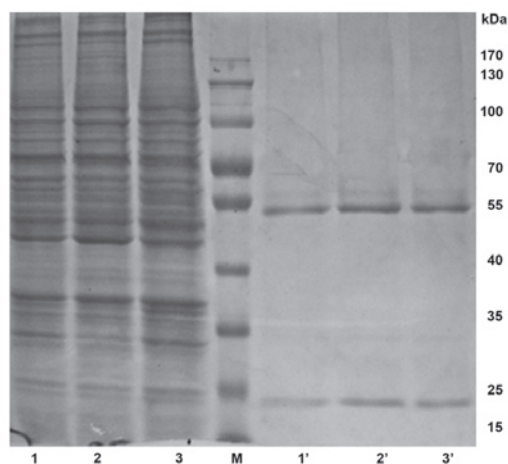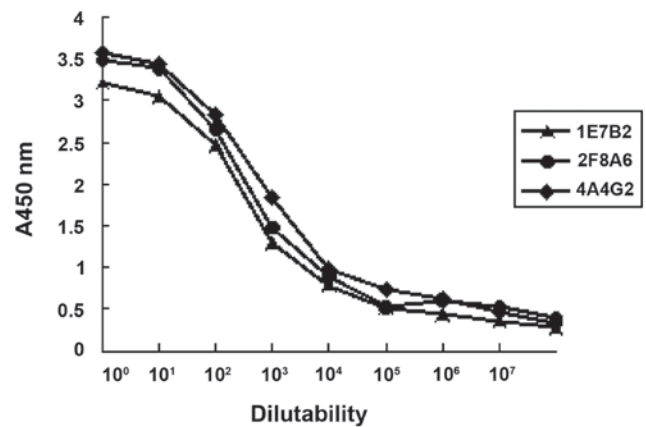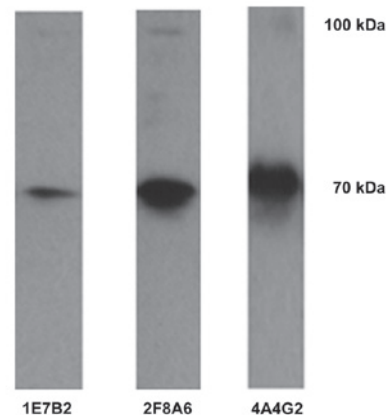
Figure 3. Purification of anti-CDB3 in mouse ascites was analyzed using 10% SDS-PAGE. The proteins were visualized by Coomassie Blue R-250 staining. Lane 1, ascitic fluid containing 1E7B2 mAb; lane 2, ascitic fluid containing 2F8A6 mAb; lane 3, ascitic fluid containing 4A4G2 mAb; M, protein molecular weight marker; lane 1', purified 1E7B2 mAb; lane 2', purified 2F8A6 mAb; lane 3', purified 4A4G2 mAb. mAb, monoclonal antibody; CDB3, receptor binding region of *Clostridium difficile* toxin B.

mAbs were successfully purified by bitterness-ammonium sulfate and protein G agarose gel electrophoresis. Following dialyzation and condensation, the purity was identified using 10% SDS-PAGE, with a purification of >90% (Fig. 3), and the concentrations of the mAbs were 27.30 μg/ml, 64.37 μg/ml and 37.36 μg/ml, respectively. The molecular weight was ~160 kDa.

Affinity of anti-CDB3 mAb. The relative affinities of the mAbs were defined by the antibody concentration at which the OD value reached half the maximal signal at the plateau stage of antigen-antibody binding. The order of relative affinity of the three selected mAbs were 4A4G2, 2F8A6, 1E7B2 (Fig. 4).

Specificity of anti-CDB3 mAb. Western blotting results demonstrated that the 1E7B2, 2F8A6 and 4A4G2 anti-CDB3 mAbs reacted specifically to the large unit of the GST-CDB3 recombinant protein cleaved by thrombin (molecular weight, ~70 kDa) and the uncleaved GST-CDB3 recombinant protein (molecular weight, ~100 kDa), however, no reaction with the tagged protein GST was observed (molecular weight, ~26 kDa) (Fig. 5).

Establishment and optimization of ds-ELISA. The anti-CDB3 mAb (4A4G2) was successfully labeled with HRP by modified sodium periodate oxidation. The ds-ELISA was and the optimal working concentration of capture antibody and HRP-labeled mAb were demonstrated to be 20 μg/ml and 1:400, respectively, as determined using chessboard titration.

Detection of TcdB in clinical stool samples using ds-ELISA. Of the 15 specimens of A^+B^+-type *C. difficile*, 11 were positive for TcdB (73.33%), detected using ds-ELISA, 13 of the 15 A^-B^+-type *C. difficile* samples were positive for TcdB (86.67%) and all of the 15 *C. difficile* specimens were negative for TcdB.

Discussion

Rapid and accurate diagnosis of CDI is essential for improving the outcomes of patients and for reducing horizontal transmission in health care facilities. There are several types of laboratory assessments aimed at detecting pathogens and their toxins in diarrheal fecal specimens, each with their own qualities and limitations. However, an optimal test for the diagnosis of *C. difficile*-associated diseases remains to be established (34,35). Curry (36) reported that the 'diagnosis of CDI remains one of the most vexing difficulties for hospital microbiology laboratories', due to the absence of a single accepted gold standard. For several years, the cell culture cytotoxicity neutralization assay (CCNA) was regarded as the standard method for the diagnosis of CDI (34) due to its correlation with PMC with a specificity >97%, and its ability to detect toxins in the stool as levels as low as 10 pg. However, the CCNA assay is expensive, lacks standardization among laboratories, requires advanced skills and cell culture facilities and has a 24-48 h turn around period. Several studies have reported toxigenic culture (TC) as the method of choice for the diagnosis of CDI, and is suggested to be the most sensitive method for CDI detection (34,35,36). Compared with TC, CCNA has only 67-79% sensitivity (36). The Society for Healthcare Epidemiology of America and the Infectious Diseases Society of America 2010 guidelines note that 'the sensitivity and specificity of stool culture followed by identification of a toxigenic isolate, as performed by an experienced laboratory provides the standard against which other clinical tests should be compared' (3). However, this method is slow and laborious, often requires 48-72 h to complete and, therefore, is unlikely to be used in clinical laboratories as the standard method for CDI diagnosis. Furthermore, successfully culturing *C. difficile*-producing toxin without detection of toxin in the diarrheal stool specimens has not been associated with poorer clinical outcomes, compared with stools negative for toxigenic *C. difficile* (35). Nucleic acid amplification tests (NAATs) are the most recent methods used for the detection of *C. difficile*. Available NAATs for the identification of *C. difficile* genes are PCR, real-time PCR, and loop-mediated isothermal amplification (37). These assessments detect the chromosomal genes encoding TcdA and TcdB, or the toxin regulatory gene directly from stool samples (38); however, despite detecting the toxin gene, they do not detect the toxin in stool samples, raising concerns over accurate diagnosis in asymptomatic carriers. In addition, the use of PCRs may increase CDI incidence rates by >50% (39). A glutamate dehydrogenase antigen assay may be used as a rapid screening method for the presence of *C. difficile* in stools. The assessment is fast (15-45 min), convenient, inexpensive and sensitive, however, it only documents the presence of *C. difficile*, not the presence of toxigenic strains (20% of *C. difficile* strains do not produce toxin) or the presence of toxin in stool (40). Commercial rapid enzyme immunoassays

have been the most widely used diagnostic assessment for the detection of TcdA and TcdB due to the fact that they are technically simple to perform, provide rapid (same day) results and are relatively inexpensive (34); however, they may lead to more false-positive test results, compared with the cytotoxicity assay, as the polyclonal antibodies against TcdA and TcdB used in immunoassays may target other antigens at the same time (41). These limitations may be solved with the use of mAbs, as they can not only improve the specificity of the method, but also decrease the number of false positive results.

A^-B^+ *C. difficile* strains causing severe infections and outbreaks have been reported to have increased significantly increased. The prevalence of A^-B^+ strains among CDI isolates varies depending on the country; in the majority of Europe and in North America, the prevalence of A^-B^+ strains has been reported to be only 0.2-8% (8,42), however, a report from Shanghai revealed that A^-B^+ strains were responsible for 33.3% of all CDI cases in China (19), which suggests that A^-B^+ strains are more prevalent in Asia. TcdB is organized into four distinct structural domains (43): The N-terminal glucosyltransferase domain (residues 1-543), the cysteine protease domain (residues 544-807), the membrane translocation (residues 808-1850) and the C-terminal oligopeptide repeat domain (residues 1851-2366). The short, homologous regions, termed combined repetitive oligopeptides, found in TcdB are more divergent and less frequent than those found in TcdA (43). TcdB is divided into three fragments, which are separately expressed: CDB1 (amino acids 1-546) contains the catalytic domain; CDB2 (amino acids 901-1750) harbors the putative transmembrane domain; and CDB3 (amino acids 1751-2366) is thought to be the receptor binding domain. Von Eichel-Streiber *et al* (44) demonstrated that anti-CDB3 exhibited a marked reaction with CDB3 and a weak reaction with CDB1, but not with CDB2, concluding that the receptor CDB3 binding domain, harboring repetitive peptide domains, was the most antigenic region of the toxin.

The present study successfully established five hybridoma cell lines (1E7B, 1F8D3, 2F8A6, 3B6F1 and 4A4G2), which stably secreted anti-CDB3 mAb, by immunizing BALB/c mice with the GST-CDB3 recombinant protein, which was characterized using indirect ELISA and western blotting. The IgG isotype was predominant [1E7B2 IgG1 (λ), 2F8A6 IgG2a (κ) and 4A4G2 IgG1 (κ)]. Indirect ELISA demonstrated that the 2F8A6 and 4A4G2 mAbs exhibited high titers of specific binding affinities for CDB3. Western blotting revealed that the 2F8A6 and 4A4G2 mAbs specifically recognized the CDB3 protein. Therefore, the purified 4A4G2 mAb was labeled with HRP and selected as the detection antibody, and the purified 2F8A6 mAb was selected as the coated antibody to establish a ds-ELISA for the detection of TcdB in clinical diarrhea stool samples. The concentration of the coated antibody was determined as 20 μg/ml, and the optimal working concentration of HRP-labeled mAb was 1:400, determined using the chessboard titration method in ds-ELISA. To the best of our knowledge, the present study is the first to describe a ds-ELISA using monospecific antibody as a capture antibody, and mAb as a detection antibody. This type of ds-ELISA offers distinct advantages: The antibodies utilized, including secondary antibodies, are homogeneous, with specificity for CDB3 and are also less likely to interact with closely associated proteins.

Cross-reactivity of secondary antibodies is also eliminated. The results of the present study indicated that the sensitivity of the ds-ELISA was 73.33% to A⁻B⁺ toxigenic *C. difficile* strains and 86.67% to A⁻B⁺ toxigenic *C. difficile* strains, with a specificity of 100% overall.

The isolation of A⁻B⁺ *C. difficile* strains producing TcdB, but not TcdA, are widespread, and are more frequent in east Asian countries. A diagnostic method capable of rapidly detecting the presence of TcdB is required for use in microbiological laboratories in order to control these strains of *C. difficile*, however, no diagnostic reagents have been available in China. The rapid diagnosis of *C. difficile* is important and guides the treatment and control of nosocomial spread of infection. To the best of our knowledge, the present study is the first to develop novel mAbs specific to CDB3, and a ds-ELISA kit with high specificity and sensitivity for the rapid detection of TcdB was established. These provide useful tools for the diagnosis of TcdB in CDI.

Acknowledgements

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