

Molecular characterization and evaluation of *Clostridium difficile* field isolates recovered from pigs with neonatal diarrhea

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During the past decade, *Clostridium difficile* has become a major cause of neonatal enteritis in pigs and a big economic threat to pig producers.^{1,2} *C difficile* is a Gram-positive to Gram-variable, spore-forming, anaerobic bacterium, and is a common inhabitant in soil, water, and the intestinal tract of various animals. It has been reported that *C difficile* can cause enteritis in pigs from newborn to two weeks of age. The affected pigs have mildly distended abdomen, diarrhea, ascites, hydrothorax, and edema of the ascending mesocolon.^{2,3} It is also known that numerous strains of *C difficile* exist and differ in pathogenicity. The virulence factors include pilus expression, mucosa adherence, capsule production, and the ability to produce toxins.³ Non-toxigenic *C difficile* strains are avirulent, while toxigenic strains are virulent and may produce toxin A (TcdA, an enterotoxin) and/or toxin B (TcdB, a cytotoxin). Toxin A and toxin B are encoded by toxin genes *tcdA* and *tcdB* respectively. Both toxin A and toxin B are known to be the primary virulence factors of *C difficile*.⁴ Toxin A can cause fluid accumulation in the intestine, while toxin B is extremely cytopathic for tissue culture cells. Other potential virulence factors of the *C difficile* have also been identified such as a binary toxin called CDT (actin-specific ADP-ribosyltransferase toxin).^{5,6} The CDT belongs to the group of clostridial binary toxins and is composed of two independent unlinked protein chains, CDTa (enzyme component), and CDTb (binding component). The CDTa and CDTb are encoded by *cdtA* and *cdtB* genes respectively. The CDTb recognizes a cell surface receptor and allows the internalization of the CDTa into cytosol. Then the CDTa catalyzes the ADP-ribosylation reaction of monomeric actin and induces a disorganization of the host cell's cytoskeleton.⁷

Although *C difficile* has been known as a major cause of neonatal diarrhea in pigs since 2000, there are very few typing studies on porcine *C difficile* strains isolated in this country. In addition to serotyping, many methods have been used to type or fingerprint human strains of *C difficile* for outbreak investigations and epidemiological

study. So far several genotypic and phenotypic schemes have been published,⁸ which include serotyping, PCR ribotyping, electrophoresis (isotope-labeled methionine PAGE), arbitrary primed-PCR (AP-PCR), immunoblotting, restriction enzyme analysis, and restriction fragment length polymorphisms (RFLP). Among the above mentioned typing methods, a modified PCR ribotyping has been widely used in the study of human strains. However, a recent report indicated that there are only four PCR ribotypes that have been found among 144 porcine *C difficile* strains isolated in this country. It has been found that only one type (type 078) is the most common PCR ribotype among the 144 strains.⁹ Therefore, the discriminatory power of the ribotyping for the porcine strains is not absolute. According to a report published by the International *Clostridium difficile* Study Group, there are some strong correlations between three typing methods (serogroup, PAGE type, and immunoblotting type). Since these three methods are all dependent on cell surface proteins, it is not surprising.⁸

In this study, some 80 *C difficile* strains isolated from pigs with neonatal diarrhea were used for the characterization and evaluation of each individual strain using Western blot to detect its toxin production and PCR assays to detect toxin A and toxin B genes as well as the binary toxin genes. A comparison of the soluble protein profile of each isolate was also made by using electrophoresis of the soluble proteins to create a dendrogram to estimate the similarity between different strains.

Isolation, identification, and characterization of *C difficile* field strains

C difficile isolations were made by culturing colon contents or rectal swabs using standard technique. Both 5% sheep blood agar and *Clostridium difficile* selective agar with 5% horse or bovine blood (CDSA) were used and incubated in anaerobic jars with anaerobic gas

generating system. Clinical specimens were inoculated directly onto CDSA plates and streaked for single colony isolation. The plates were incubated anaerobically at 37°C for 48 hours. All isolated strains were examined by a series of PCR assays for identification and toxigenic type characterization. Briefly, a bacterial colony was taken from the culture plate and suspended in 100 µl of sterile deionized water in a microcentrifuge tube. The sample was boiled for 5 minutes and centrifuged for 3 minutes at 2,000 × g. The supernatant was used as a DNA template in PCR assay.

Amplification of *tpi* gene for *C difficile* identification

The *tpi*-specific primers, *tpi-F* (5'-AAAGAAGCTACTAAGGGTACAAA-3') and *tpi-R* (5'-CATAATATTGGGTCTATTCCTAC-3'), were used to generate a 230-bp amplified fragment specific for *C difficile* as described before.¹¹ This *tpi* (triose phosphate isomerase) house keeping gene had been used to differentiate *Clostridium* species and the 230-bp DNA amplicon is used as a species-specific marker for *C difficile*. PCRs were performed on a BIO-RAD C1000 thermocycler in a final volume of 25 µl containing 10 µl of DNA template, 10% (vol/vol) glycerol, 0.5 µM *tpi-F*, 0.5 µM *tpi-R*, 200 µM each dNTP, 0.5 U of Taq DNA polymerase, in 2.5 µl of 10X PCR buffer. The PCR mixtures were denatured at 95°C for 3 minutes, and then a touch down procedure was done, consisting of 30 seconds at 95°C, annealing for 30 seconds at temperatures decreasing from 65°C to 55°C during the 11 cycles (with 1°C decremental steps in cycle 1 to 11), and a final extension step at 72°C for 30 seconds. A total of 40 cycles were performed. Twelve microliters of PCR products were run on a 2% agarose gel stained with ethidium bromide.¹⁰

Amplification of *tcdA* and *tcdB* genes for large *Clostridium* toxin genes detection

The *tcdA*-specific primers (5'-GCATGATAAGGCAACTTCAGTGG-3', 5'-GAGTAGTTCCTCCTGCTCCATCAA-3') were used to generate a 600-bp DNA amplicon. The *tcdB*-specific primers (5'-GGTGGAGCTTCAATTGGAGAG-3', 5'-GTGTAACCTACTTTCATAACACCAG-3') were used to generate a 399-bp DNA amplicon. PCRs were performed in a final volume of 50 µl containing 5 µl of DNA template, 5 µl of 10XPCR buffer, 16 µl of dNTP (2.5 mM each), 0.50 µl of each primer (40 µM each), 2 U of Taq DNA polymerase, 4 µl of magnesium

chloride (25 mM), in 18.6 µl deionized water. The PCR mixtures were denatured at 94°C for one minute, and 40 cycles at 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for one minute, and then one cycle at 4°C and soak. The PCR products were run on a 2% agarose gel stained with ethidium bromide.¹¹

Amplification of *cdtA* and *cdtB* genes for binary toxin genes detection

The *cdtA*-specific primers (5'-TGAACCTGGAAAAGGTGATG-3', 5'-AGGATTATTATTACTGGACCATTTG-3') were used to generate a 353-bp DNA amplicon. The *cdtB*-specific primers (5'-CTTATTGCAAGTAAATACTGAG-3', 5'-ACCGGATCTCTTGCTTCAGTC-3') were used to generate a 490-bp DNA amplicon. PCRs were performed in a final volume of 50 µl containing 5 µl of 10XPCR buffer, 5 µl of DNA template, 0.5 µl of dNTP (10 mM each), 1.5 µl of each primers (50 µM each), 6 µl of 50 mM magnesium chloride, 1 U of Taq DNA polymerase, 5 µl of 1% Triton X-100 in 25.3 µl of de-ionized water. The PCR amplifications were performed with 30 cycles of 94°C for 45 seconds, 52°C for 60 seconds, and 75°C for 80 seconds. The PCR products were run on a 2% agarose gel stained with ethidium bromide.⁵

Differentiation based on soluble protein profile (SPP) analysis

Each field strain was grown on a 5% sheep blood agar and incubated at 37°C in an anaerobic jar with anaerobic gas generating system for 48 hours. Bacterial colonies on each plate were harvested with a sterile cotton swab and transferred to a microcentrifuge tube containing 1 ml of sterile PBS. The tube was centrifuged at 10,000 X G for 3 minutes and the PBS was removed. The cell pellet was suspended in 500 µl of a Bacterial Cell Lysis/Extraction Reagent (Sigma Chemical B3678) at 4°C for an hour and then was centrifuged again at 10,000 X G for 3 minutes. The protein concentration of the supernatant was determined and the supernatant was used for gel electrophoresis. Each protein sample was adjusted to the same concentration using PBS and loaded on an Invitrogen's NuPAGE Novex Bis-Tris Gel after mixing with loading buffer and heated in a 90°C water bath for 10 minutes as indicated by the manufacturer. After electrophoresis, the gel was stained with the Colloidal Blue Staining Kit (Invitrogen LC6025). The gel documentation was performed with BIO-RAD's Molecular Imager Gel Doc XR System and the dendrogram was created using BIO-RAD's Quantity One 1-D analysis software.

AP-PCR typing

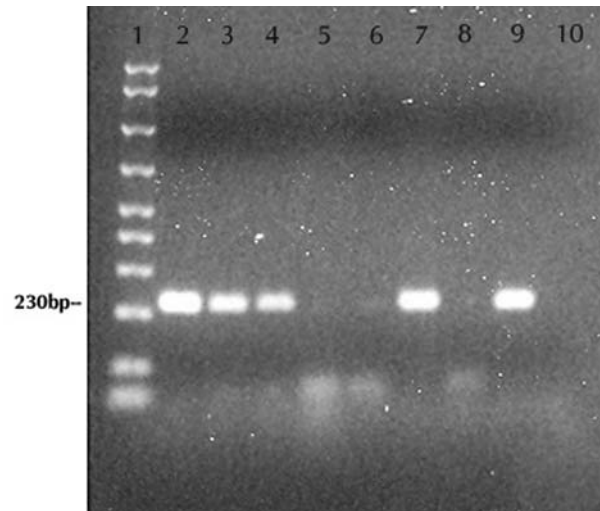
The PCR conditions used were as described before.¹⁴ Amplification was conducted in a volume of 25 μ l containing 2.5 μ l of 10X PCR buffer, 0.5 μ l of 0.005% gelatin, 1.0 μ l of dNTP (25 mM each), 0.2 μ l of 100 μ M ARB11 primer (5'-CTAGGACCGC-3'), 0.4 μ l of Taq polymerase (5U/ μ l), 5.0 μ l of DNA, 2.0 μ l of 50 mM magnesium chloride, and 13.4 μ l of deionized water. Samples were amplified on a thermocycler for 45 cycles at 95°C for one minute, 36°C for one minute, and 72°C for two minutes. Fifteen microliters of PCR product mixed with 3 μ l of loading buffer was loaded on a 1.5% agarose gel and electrophoresed at 200 V until the dye front had migrated for 10 cm. The gel was stained with ethidium bromide and gel documentation was performed as described above.

Immunoblot analysis of TcdA and TcdB in the bacterial culture

Undiluted bacterial culture supernatant was electrophoresed as described above and transferred to nitrocellulose and detected with antibodies against TcdA and TcdB. If a test sample was determined to be positive for the toxins, a series of ten fold dilution of the test sample was tested again to determine its endpoint.

In this study we attempted to identify *C difficile* field strain using a species-specific PCR. Among the 80 isolates that were first suspected to be *C difficile* based on colony morphology and Gram stain, 72 isolates were identified to be *C difficile* by a species-specific PCR while 8 isolates were not *C difficile*. The species-specific internal fragment of the *tpi* gene was detected as shown in Figure 1. The prevalence of the *tcdA*, *tcdB*, *cdtA*, and *cdtB* toxin genes among the 72 field isolates were also revealed by PCR assays as shown in Table 1 and Table 2. About 70% of the field isolates carry both toxin A and toxin B genes, while around 11% of the isolates carry just one of the two large clostridium toxin genes (Figure 2). It was known that clinical isolates from sick animals usually carry both toxin A and toxin B genes. However this study also reveals that an increased number of clinical isolates carry just one toxin gene. This finding was in agreement with some recent reports that indicated an increasing number of severe *C difficile* infection in human was due to toxin A negative and toxin B positive strains.^{12,13} Large clostridium toxin genes negative, binary toxin genes positive strains had been isolated from symptomatic and asymptomatic patients in human cases.¹⁵ In this study we did not isolate any *C difficile* strains that were negative for large clostridium toxin genes and positive for binary toxin gens.

Figure 1: Identification of *C difficile* using a species-specific PCR to amplify a *tpi* gene. Lane 1: BIO-RAD AmpliSize Molecular Ruler 50-2000 bp ladder; lane 2, 3, 4, and 7: test samples positive for the 230 bp *tpi* gene and were identified as *C difficile*; lane 5, 6, and 8: test samples negative for *tpi* gene and were determined to be not *C difficile*; lane 9: *C difficile* ATCC 43255 as positive control; lane 10: *C perfringens* as negative control.



However, we did isolate 17 *C difficile* strains that were positive for toxin A, toxin B, and binary toxin genes. Further study is needed to determine if the binary toxin can act as a virulence factor or just act as an adjunctive virulence factor in *C difficile* field strains.

The test schemes adopted in this study allow us to distinguish *C difficile* from other *Clostridium* species and also distinguish *C difficile* field strains between the four main toxigenic types (Table1). The use of PCR assay for the binary toxin genes (Figure 3) further help distinguish the field strains between six toxigenic types (Table2). In addition to toxigenic typing, AP-PCR typing and SPP typing will give each field isolate a unique fingerprinting and can be used for the intra-species differentiation. The soluble protein profile of eight *C difficile* field strains was shown in Figure 4 and their SPP dendrogram was shown in Figure 5. The AP-PCR banding patterns of the five *C. difficile* strains were shown in Figure 6 and the AP-PCR dendrogram was shown in figure 7. In addition to SPP dendrogram, AP-PCR dendrogram will give us one more approach to the intra-species differentiation of *C difficile*. The detection of toxin A and toxin B in the culture supernatant by Western blot using monoclonal antibody will allow us to evaluate the potential of each isolate in its toxin production in vitro. As shown in Figure 8, monoclonal antibody specific for

Table 1: Prevalence of toxin A and toxin B genes among 72 *C difficile* strains

Toxigenic type	tcdA	tcdB	Number of isolates	Percentage
A+B+	+	+	50	69.5%
A+B-	+	-	2	2.8%
A-B+	-	+	6	8.3%
A-B-	-	-	14	19.4%

Table 2: Prevalence of toxin A, toxin B, and binary toxin genes among 72 *C difficile* strains

Toxigenic type	Large toxin		Binary toxin		Number of isolates	Percentage
	tcdA	tcdB	cdtA	cdtB		
A+B+CDT+	+	+	+	+	17	23.6%
A+B+CDT+	+	+	+	-	20	27.8%
A+B+CDT-	+	+	-	-	13	18.1%
A+B-CDT-	+	-	-	-	2	2.8%
A-B+CDT+	-	+	+	-	6	8.3%
A-B-CDT-	-	-	-	-	14	19.4%

Figure 2: Detection of toxin A and toxin B genes using PCR assay. Lane 1: BIO-RAD Ampli-Size Molecular Ruler 50-2000 bp ladder; lane 2: positive control with a known toxigenic type (A+B+) was amplified with *tcdA* primers to generate a 600 bp DNA amplicon ; lane 3: test sample A that was amplified with *tcdA* primers and was found to be positive for *tcdA* gene; lane 4: test sample B that was amplified with *tcdA* primers and was found to be negative for *tcdA* gene; lane 5: positive control with a known toxigenic type (A+B+) was amplified with *tcdB* primers to generate a 399 bp DNA amplicon; lane 6: test sample A that was amplified with *tcdB* primers and was found to be positive for *tcdB* gene; 7: test sample B that was amplified with *tcdB* primers and was found to be positive for *tcdB* gene.

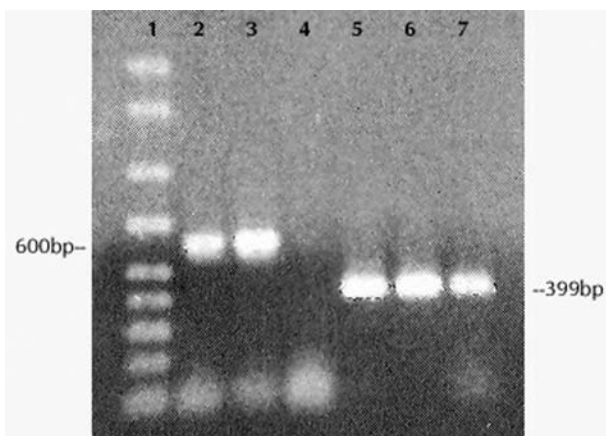
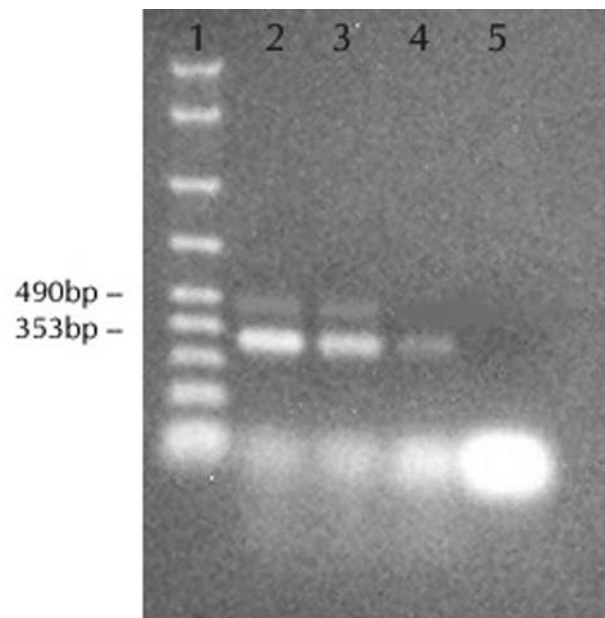


Figure 3: Detection of the binary toxin genes using PCR assay. Lane 1: BIO-RAD AmpliSize Molecular Ruler 50-2000 bp ladder; lane 2 and 3: test samples that were positive for both *cdtA* and *cdtB* genes; lane 4: test sample that was positive for *cdtA* gene and negative for *cdtB* gene; lane 5: test sample that was negative for both *cdtA* and *cdtB* genes.



toxin A will detect a 52 kD toxin A or detect one of the two dissociated subunits of toxin A (41.5 kD and 16 kD).¹⁶ Based on the above mentioned test schemes, all of the *C difficile* strains isolated from the same farm at the same time or at different time can be characterized and evaluated. All of the information will be helpful for the swine practitioners in designing a more effective autogenous vaccine to prevent *C difficile* infection in neonatal pigs.

Figure 4: SDS-PAGE of the soluble proteins of eight *C difficile* strains and stained with Colloidal Blue. Lane 1 and 10: BIO-RAD Precision Plus Protein Standards; lane 2, 3, 4, 5, 6, 7, 8, and 9: soluble proteins of eight *C difficile* field strains.

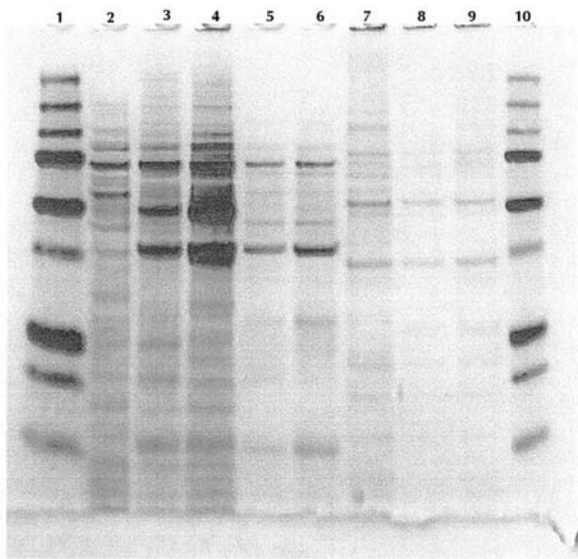


Figure 5: SPP dendrogram to show the similarity (%) in soluble protein profile (SPP) among eight *C difficile* field strains.

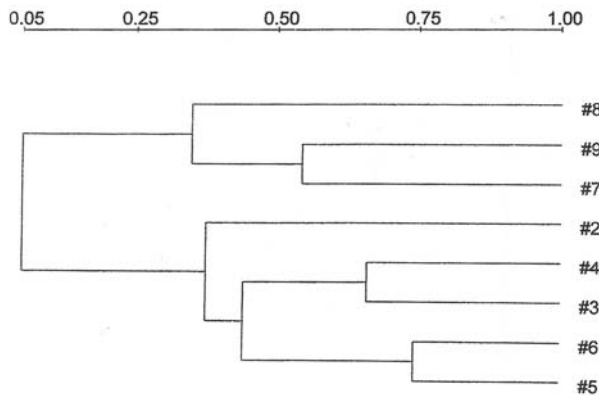


Figure 6: AP-PCR banding patterns of *C difficile* isolates. Lane 1: BIO-RAD AmpliSize Molecular Ruler 50-2000 bp Ladder; lane 3 to 7: five strains of *C difficile*.

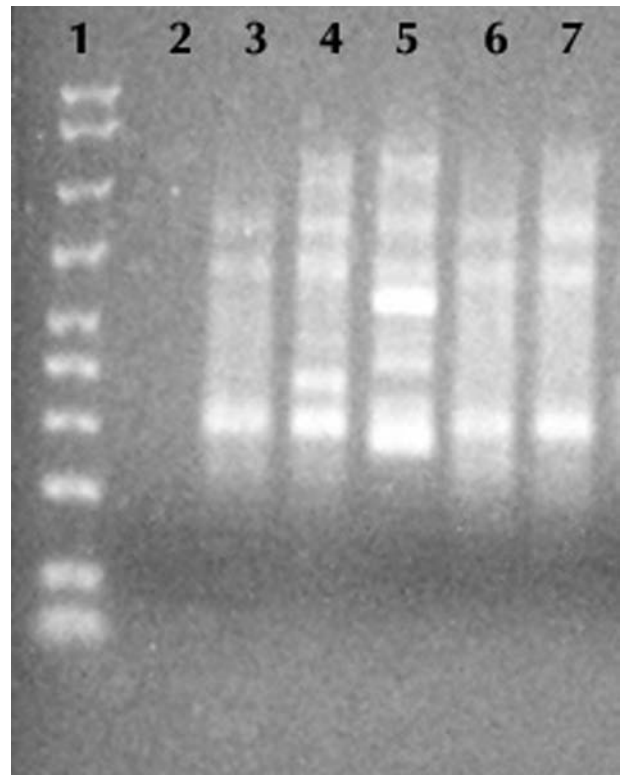


Figure 7: AP-PCR dendrogram to show the similarity (%) in DNA banding patterns among five *C difficile* field strains.

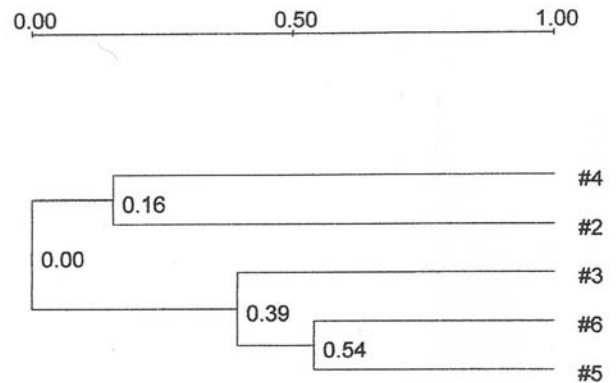
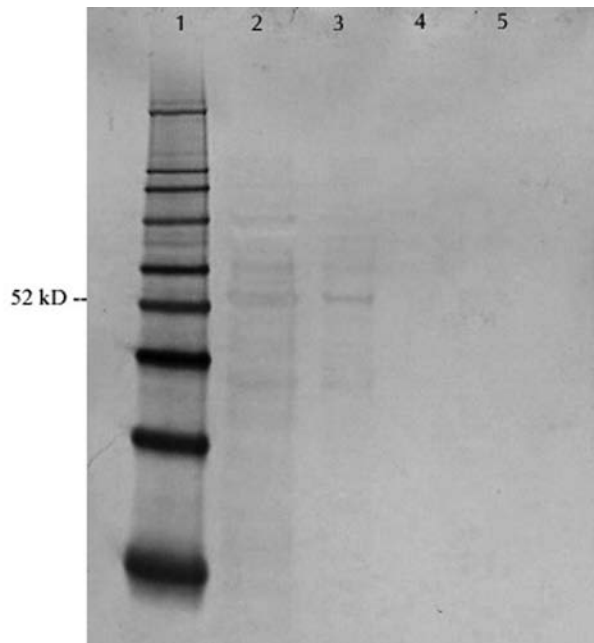


Figure 8: Detection and quantification of toxin A in *C. difficile* culture supernatant using Western blot analysis with a monoclonal antibody against toxin A. Lane 1: Molecular weight markers (Invitrogen's MagicMark XP Western standard); lane 2: undiluted culture supernatant of a test sample; lane 3: 1:10 diluted supernatant of a test sample; lane 4: 1:100 diluted supernatant of a test sample; lane 5: 1:1,000 diluted supernatant of a test sample.



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