Published in final edited form as:

Diagn Microbiol Infect Dis. 2010 February ; 66(2): 140. doi:10.1016/j.diagmicrobio.2009.09.014.

Development of a microarray for identification of pathogenic *Clostridium* species

Tavan Janvilisri^{1,4}, Joy Scaria¹, Robin Gleed², Susan Fubini², Michelle M. Bonkosky³, Yrjö T. Gröhn¹, and Yung-Fu Chang^{1,*}

¹ Department of Population Medicine and Diagnostic Sciences, College of Veterinary Medicine, Cornell University, Ithaca, NY USA 14853

² Department of Clinical Sciences, College of Veterinary Medicine, Cornell University, Ithaca, NY USA 14853

³ Oak Ridge Institute for Science and Education, P.O. Box 117, Oak Ridge, TN USA 37831-0117

⁴ Department of Biology, Faculty of Science, Mahidol University, Bangkok, Thailand 10400

Abstract

In recent years, *Clostridium* species have rapidly reemerged as human and animal pathogens. The detection and identification of pathogenic *Clostridium* species is therefore critical for clinical diagnosis and antimicrobial therapy. Traditional diagnostic techniques for clostridia are laborious, time-consuming and may adversely affect the therapeutic outcome. In this study, we developed an oligonucleotide diagnostic microarray for pathogenic *Clostridium* species. The microarray specificity was tested against 65 *Clostridium* isolates. The applicability of this microarray in a clinical setting was assessed with the use of mock stool samples. The microarray was successful in discriminating at least four species with the limit of detection as low as 10⁴ CFU/ml. In addition, the pattern of virulence and antibiotic resistance genes of tested strains were determined through the microarrays. This approach demonstrates the high-throughput detection and identification of *Clostridium* species and provides advantages over traditional methods. Microarray-based techniques are promising applications for clinical diagnosis and epidemiological investigations.

INTRODUCTION

Members of the genus *Clostridium* are anaerobic spore-forming rod-shaped Gram-positive bacteria. They exist as free-living bacteria as well as pathogens that infect both humans and animals. Most pathogenic *Clostridium* species are known to produce toxins, which are responsible for a wide range of diseases (Hatheway, 1990). Examples include (i) *C. botulinum* produces neurotoxins that cause botulism, a disease characterized by a symmetrical, descending paralysis (Sobel, 2005); (ii) *C. difficile* is a major nosocomial pathogen that can cause a spectrum of symptoms from mild diarrhea to severe pseudomembranous colitis (Bartlett and Gerding, 2008); (iii) *C. perfringens* is a common cause of food-poisoning and its toxins can also cause gas gangrene (Brynstad and Granum, 2002); and (iv) *C. tetani* produces a potent biological toxin that is the causative agent of tetanus (Brook, 2008). Other

*Corresponding author: Department of Population Medicine and Diagnostic Sciences, College of Veterinary Medicine, Cornell University, Ithaca, New York 14853 USA; Phone: (607) 253-2675. Fax: (607) 253-3943., yc42@cornell.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Clostridium species such as *C. acetobutylicum* and *C. thermocellum* are useful in the fermentation industry (Papoutsakis, 2008).

Conventional methods for detection of pathogenic *Clostridium* in clinical and food samples are based on culture techniques (Peterson et al., 1996), tissue cell culture cytotoxicity (Delmee et al., 2005) or mouse bioassays (Lindstrom et al., 2001). However, these assays are extremely time-consuming as they require several days to obtain complete results. As a consequence, these methods are not suitable for high-throughput analyses and the affected patients may not receive optimal treatment. Several laboratories have established more rapid, sensitive and specific assays for the diagnosis of pathogenic *Clostridium*. Enzyme immunoassays have been developed and some are commercially available for detection of clostridial toxins such as neurotoxins of *C. botulinum* (Lindstrom et al., 2001), alpha and beta toxins of *C. perfringens* (Asha and Wilcox, 2002), and TcdA/B of *C. difficile* (Snell et al., 2004). These immunoassays are usually not as sensitive as traditional methods and often produce false-negative results (Turgeon et al., 2003). However, certain cases with culture-positive toxin-negative results are not necessarily clinical false-negatives since a positive culture that is not toxinogenic would not be clinically significant. The utility of PCR-based techniques with specific genes of *Clostridium* species has also been explored. Several studies have demonstrated the use of multiplex PCR for detection of *Clostridium* species (Lindstrom et al., 2001; Gurjar et al., 2008; Persson et al., 2008). However, in the complicated diagnostics such as the cases with closely related species, a larger pool of primers is needed in order to identify more target pathogens and the PCR sensitivity often decreases, pointing to the major drawback of these PCR-based assays.

In recent years, the complete genome sequences of several pathogenic bacteria have been published. These data provide opportunities for the development of microarray-based pathogen diagnostic assays that offer several advantages over classic diagnostic assays (Bodrossy and Sessitsch, 2004; Mikhailovich et al., 2008). Microarrays provide higher specificity than PCR-based methods, which are based only on a single gene/protein. They also have the capability to analyze thousands of target sequences in a short period of time. However, the pitfall of diagnostic microarrays is the higher cost and skilled labor that is needed to standardize the protocols and optimize hybridization conditions, which are the key factors affecting the discriminatory power of this assay. It is also possible that the sensitivity of microarrays may be compromised compared to that of single-plex PCR. Nevertheless, given the nature of information that can be gleaned from one single assay and the plummeting cost of arrays each year, they are becoming more popular. It will also be possible to perform cross comparisons of datasets from various sources as more data become available through public databases such as Gene Expression Omnibus (Barrett and Edgar, 2006). Another bottleneck for the wide implementation of diagnostic arrays was the lack of user-friendly software for data analysis (Loy and Bodrossy, 2006). At present, more online and offline programs are becoming freely available (Dudoit et al., 2003; Scaria et al., 2008b).

The genomes of many *Clostridium* species have been reported and deposited in a public database. These include *C. acetobutylicum*, *C. beijerinckii*, *C. botulinum*, *C. cellulolyticum*, *C. difficile*, *C. kluyveri*, *C. novyi*, *C. perfringens*, *C. phytofermentans*, *C. tetani*, and *C. thermocellum* (from the Genome Database, Website: <http://www.ncbi.nlm.nih.gov/sites/entrez?db=genome>). In this study, we constructed oligonucleotide-based microarrays, which included probes specific to (i) *Clostridium* species-specific genes; (ii) clostridial virulence genes; and (iii) antibiotic resistance genes conferring resistance to various classes of antibiotics. These features allow us to identify *Clostridium* species and to further characterize the tested strains in regard to their virulence and antibiotic resistance. The practicability, specificity and sensitivity of our microarray for the identification and characterization of *Clostridium* species was evaluated with reference strains, clinical

isolates and simulated fecal samples. Here, we demonstrate the applicability of this microarray as a diagnostic tool for *Clostridium* species.

MATERIALS AND METHODS

Bacterial strains and culture conditions

Clostridium strains used in this study are listed in Table 1. All the isolates were cultivated in pre-reduced anaerobically sterilized peptone yeast extract broth with glucose (Anaerobe Systems, Morgan Hill, CA) at 37 °C for 48 h under anaerobic conditions.

Microarray probe design and array fabrication

All the bacterial genomes were downloaded from RefSeq release 32 (The National Center of Biotechnology Information; NCBI. Website: <ftp://ftp.ncbi.nih.gov/refseq/release/>). Species-specific genes were selected using BLAST-P at 10% identity cutoff and 1e-5 E value. At this cutoff value, genes with no hits in other species were considered species-specific genes. rRNA genes were retrieved from the whole genome sequences based on the genome annotation. Virulence-associated genes were downloaded from virulence factors of bacterial pathogens database (VFDB) (Yang et al., 2008). The beta-lactam and vancomycin resistance genes were retrieved from antibiotic resistance genes online (ARGO) (Scaria et al., 2005). Other categories of antibiotic resistance genes were retrieved from GenBank using NCBI E-utilities based queries.

Probes were designed using Agilent eArray algorithm (<https://earray.chem.agilent.com/earray/>). A tiling design with a minimum distance of 40 bases between the probes was used for rRNA (positive control), virulence and antibiotic resistance genes. To accommodate the possible variations in any given region of the probe, four probes for each rRNA gene and five probes for each antibiotic resistance gene were selected to cover different regions of the genes. For species-specific genes, one probe per gene was designed to bind to the most unique region of the sequence. All probes were designed to have ~ 60 bases with a melting temperature of 80 °C. The arrays also contained the quality control gridline with bright and dark corner probes. to test the effectiveness of the hybridization system and served as a reference coordinate for scanning. In the final array, each of the probes was printed in triplicate using Agilent SurePrint platform (Agilent Technologies, Santa Clara, CA). All of the oligonucleotide probes are listed in Table S1.

DNA isolation and labeling

Prior to the genomic DNA extraction and array hybridization, all the strains were blind-coded to verify the stability and specificity of the microarray. DNA isolation and labeling was performed as previously described (Janvilisri et al., 2009). Briefly, genomic DNA was extracted and purified using DNeasy Blood and Tissue Mini Kits (Qiagen, Valencia, CA) following the manufacturer's instructions for gram-positive bacteria. The concentration of DNA and its purity were determined by UV spectrophotometry (Beckman Coulter, Fullerton, CA.). Genomic DNA from each isolate was labeled by an enzymatic chemical-labeling method. Genomic DNA (2 µg) was fragmented by *DraI* digestion at 37°C for 3 h and purified using a QIAquick PCR Purification Kit (Qiagen). Following the digestion, 10 µg of exo-resistant random primers (Fermentas, Glen Burnie, MD) was added to the mixture, which was denatured at 95°C for 10 min. The sample DNA was labeled with 25 U of Klenow fragment (New England Biolabs, Ipswich, MA), dNTP mix (0.12 mM each dATP, dCTP, and dGTP and 0.03 mM dTTP), and 0.1 mM of Cy3-dUTP (Amersham Biosciences, Piscataway, NJ) at 37°C for 24 h. Unbound dye was removed from the DNA using a QIAquick PCR Purification Kit (Qiagen). The efficiency of dye incorporation and the labeled sample yields were monitored by UV spectrometry as previously described (Palaniappan et al., 2006; Scaria et al., 2008a).

Microarray hybridization

Hybridization of Cy3-labeled DNA to the microarray was carried out according to the Agilent Oligonucleotide Array-Based CGH Protocol with some modifications. Briefly, 16 µl of the Cy3-labeled DNA was mixed with 22.5 µl of 2× hybridization buffer, 4.5 µl of 10× blocking agent, 1 mg/ml salmon sperm DNA, and 1 mg/ml yeast tRNA. The mixture was heated to 95°C for 3 min and immediately incubated at 37°C for 30 min. The mixture was then transferred onto the microarray and hybridized for 24 h at 42°C in a hybridization chamber (Agilent). After hybridization, unbound fluorescent fragments were washed away with aCGH wash buffer A at room temperature for 5 min and aCGH wash buffer B (Agilent) at 37°C for 1 min. The slides were then dried and scanned immediately to minimize impact of environmental oxidants on signal intensities. Hybridizations were carried out in duplicate for each isolate. All the experiments were performed by one person and invalid hybridizations were repeated before data analysis.

Data analysis

Arrays were scanned using an Agilent G2565AA microarray scanner, with excitation at 540 nm and emission at 570 nm (Cy3) at 5-µm resolution. Scanned images were uploaded as TIFF files onto the Feature Extraction Software v10.1.1.1 (Agilent) for analysis of fluorescent intensity. The local background value was subtracted from the intensity of each spot. Spot parameters including morphology of spots and background signals were considered, and poor spots were flagged for elimination from the analysis. All subsequent data analyses were performed using Microsoft Excel and Microbial Diagnostic Array Workstation (Scaria et al., 2008b) to determine the presence or absence of these genes. The mean $\log_2$ of the tester signal and standard deviation (S.D.) were calculated from all replicates (at least two slides each with three spots for a single probe). The spots with the mean $\log_2$ values greater than the average $\log_2$ of positive control signals minus $3 \times \text{S.D.}$ were considered to be positive. The cutoff values for these parameters were empirically determined in pilot experiments and used to tag spots either as positive or negative. The hybridization data were visualized and clustered using various algorithms in Avadis Prophetic 3.3 software (Strand Genomics, Union City, CA) (Palaniappan et al., 2006; Scaria et al., 2008a).

Sensitivity and specificity test

A fresh culture of *C. difficile* strain 630 was serially diluted from 10^8 to 10^2 CFU/ml. The spiked samples were then prepared by adding 1 ml of different concentrations of *C. difficile* 630 to ~0.3 g of fresh stool samples from healthy horses. DNA from stool was extracted using the QIAamp DNA Stool Mini kit (Qiagen). In addition, four *Clostridium* species were added to stool samples at 10^8 CFU/ml in order to test the specificity and capacity of our diagnostic arrays to discriminate among species. Hybridizations were performed in duplicate for each condition.

RESULTS

Oligonucleotide probe design and microarray construction

The primary objective of this research project was to develop an oligonucleotide microarray for diagnosis of pathogenic *Clostridium* species using a single gene chip. Our microarray-based method required an initial probe selection step, which was performed to identify species-specific targets using the gene sequences from RefSeq release 32 (NCBI). Each diagnostic microarray contained four probe categories. Group I consisted of 260 probes targeting the portions of *Clostridium* rRNA genes, providing a benchmark for positive controls. Group II comprised probes that were specific to each *Clostridium* species. There were 72 specific probes for *C. boltae*, 51 for *C. botulinum*, 64 for *C. difficile*, 155 for *C. leptum*, 40 for *C. novyi*, 28 for

C. perfringens, and 43 for *C. tetani*. Group III contained 109 oligonucleotide probes corresponding to known clostridial virulence factors such as genes related to toxin production and host cell attachment. Group IV contained a set of 3,000 probes that were designed on a spectrum of antibiotic resistance genes. Multiple target regions were selected for each gene to increase the confidence of the analysis. All oligonucleotide probes used in this study are listed in the supplemental material (Table S1). The probes were *in situ* synthesized and arrayed onto glass slides.

Microarray specificity and sensitivity

The performance of the microarray was tested with a collection of 65 clinical isolates and reference strains of four target species as well as other related clostridia (Table 1). Initial analysis of the data from negative strains, positive reference strains and a blank control allowed us to identify the threshold value for positive signals. Every spot showing a $\log_2$ intensity value higher than the mean of $\log_2$ intensity of all the rRNA probes minus $3 \times \text{S.D.}$ was considered a positive signal. The $\log_2$ intensity values of all tested strains are listed in Table S2. The hybridization results and hierarchical clustering of the tested isolates are shown in Fig. 1. At the selected cutoff, all the rRNA gene probes were positive for the tested strains, assuring that these probes were suitable for use as positive controls. Microarray results with the genomic DNA from *C. difficile*, *C. novyi*, *C. perfringens*, and *C. tetani* isolates revealed specific hybridization with the species-specific gene probes and cross-hybridization of these probes was not observed. The clear clustering of these species from the microarray results indicated a high degree of confidence in specificity of the probes (Fig. 1). The probes specific to *C. botatae*, *C. botulinum*, and *C. leptum* were not tested for specificity due to the lack of strain availability, however, our results showed no non-specific binding to these probes. The sensitivity of our microarray was evaluated using mock stool specimens. A serial dilution from 10^8 to 10^2 CFU/ml of *C. difficile* 630 was added to fresh stool specimens from healthy horses. Then, DNA was extracted and hybridized with the oligonucleotide probes on the microarrays. The $\log_2$ intensity values of all conditions are listed in the supplementary material (Table S2). All probes specific to *C. difficile* were positive at levels as low as 10^4 CFU/ml. Some *C. difficile*-specific probes were found to be positive at 10^3 CFU/ml but none was detected below this concentration. Furthermore, we evaluated the ability of the microarray to identify *Clostridium* species from a mixed bacterial population of *C. difficile*, *C. novyi*, *C. perfringens*, and *C. tetani*. The resulting hybridization signals revealed that the microarray could efficiently and specifically identify all species present in the mixture.

Characterization of virulence and antibiotic-resistance genes

Apart from the species-specific probes, we also included probes to detect genes associated with virulence and antibiotic resistance to further characterize the tested isolates. The hybridization patterns of virulence gene probes are shown in Fig. 2. The obtained results were verified using the available sequenced strains (CD1, CD8, CN, CP1, CP2 and CP3). For *C. difficile* isolates, our results are consistent with previous publications (Stabler et al., 2006; Janvilisri et al., 2009) and our data that all *C. difficile* strains contained fibronectin binding protein (CD2592). Their toxin production profiles also matched the background information. Interestingly, fibronectin-binding proteins were also found in *C. novyi* (NT01CX_1954), *C. perfringens* (CPE1847, CPF_2101, and CPR_1815), and *C. tetani* (CTC01606). Most of the tested strains contained hemolysins and sialidases, which play a role in bacterial nutrition as well as pathogenesis. The pattern of presence or divergence of genes responsible for the resistance to aminoglycoside, beta-lactam, chloramphenicol, tetracycline, and vancomycin are highlighted (Fig. 3). It is noteworthy that most of the genes, whose signals were positive, were related to extrachromosomal elements. The contribution of extrachromosomal elements to clostridial pathogenic phenotypes has been reviewed (Bartlett and Gerding, 2008). It is well known that *C. difficile*-associated diseases can be established when patients receive antibiotics. Standard

management is to withdraw the implicated antibiotic and treat with oral vancomycin (Gerding et al., 2008). Our results revealed that not all *C. difficile* isolates possess vancomycin resistance genes, providing evidence that vancomycin is effective for the treatment of *C. difficile* infection.

DISCUSSION

The application of microarray-based methods in the context of clinical diagnostics has been extensively demonstrated. These assays are gaining popularity due their ability to hybridize to thousands of gene targets for the simultaneous detection of multiple bacterial pathogens in a single diagnostic test (Loy and Bodrossy, 2006). This research represents another example of the application of oligonucleotide microarrays in the detection and identification of pathogenic *Clostridium* species. Our strategy in the design of the microarray was to target 4 types of genes: (i) rRNA genes, corresponding to the conserved consensus genes among *Clostridium* species; (ii) species-specific genes, aiming to detect the unique genes in each species; (iii) virulence-associated genes from *Clostridium* species; and (iv) antibiotic resistance genes. The rRNA gene probes were used to provide a within-array positive control for choosing a statistical threshold for classifying probes as present or absent. This is similar to the probes for house-keeping genes, whose expressions remain unchanged in transcriptome arrays. Formerly, it was not easy to add or remove gene probes from the custom spotted arrays. The new generation SurePrint technology allows users to add and/or remove probes on any given chip. This makes our design highly flexible as the panel of oligonucleotide probes can be easily expanded to include sequences for additional species, virulence, and antibiotic resistance determinants as they become more available. Compared to PCR-based arrays, oligonucleotide-based microarrays provide technical advantages such as a lower rate of cross-hybridization, higher specificity, and better probe concentration control (Lyons, 2003). Although the short oligonucleotides < 35 nt have been used in several diagnostic microarrays, especially for detecting genetic variants in target genes in a bacterial population (Hacia and Collins, 1999), here we designed longer oligonucleotide probes (~60 bases), which enhanced specificity and reduced the probability of misidentification.

Our version of a *Clostridium* diagnostic microarray allows simultaneous species identification as well as detection of important virulence and antibiotic resistance genes in a single assay. We demonstrated that our microarrays successfully identified all 29 strains of *C. difficile*, *C. novyi*, *C. perfringens* and *C. tetani* and discriminated them from 36 isolates of other *Clostridium* species. In general, there are > 10¹¹ bacterial cells of over 400 bacterial species per gram of clinical sample content (Carman et al., 1993). The results from microarrays with the mock stool specimens showed the feasibility of identifying and characterizing several *Clostridium* species with a sensitivity as low as 10⁴ CFU/ml. Furthermore, our microarrays also shed light on the possible virulent and antibiotic resistant phenotypes of tested strains. The observations on antibiotic resistance gene patterns among *Clostridium* strains provide the first clue to their antibiotic resistant traits. The results revealed heterogeneity within each species. A number of positive probes in the antibiotic resistance category are associated with extrachromosomal elements, suggesting that these elements may contribute to the spread of antibiotic resistance among *Clostridium species* (Bruggemann, 2005). The antibiotic susceptibility assays may be performed to evaluate the correlation between the genotypic and phenotypic features of each strain.

Traditional methods for the diagnosis of *Clostridium species* in most clinical laboratories rely on culturing, cytotoxicity testing, biochemical reactions, antibody-based toxin detection, and PCR-based assays. Although these tests are widely used and found to be satisfactory to a certain extent, they have several limitations such as the long turnaround time for culturing and cytotoxicity testing (Peterson et al., 1996; Lindstrom et al., 2001; Delmee et al., 2005), high

cost of antibody production and lower sensitivity for immunoassays (Turgeon et al., 2003). PCR-based methods have been developed in order to improve sensitivity and specificity to detect and differentiate pathogenic species. However, simultaneous analysis of multiple genetic characteristics of target pathogens in a single test has proven to be difficult using PCR. The microarray-based diagnostics therefore has significant advantages over the aforementioned methods. It is a rapid, sensitive, specific and high-throughput assay that allows parallel detection of large number of genes in a single experiment. The high-throughput feature of the arrays is particularly useful in the detection and analysis of outbreak strains. In such instances, simultaneous detection as well as antibiotic resistance and virulence profiles revealed by microarrays will be extremely valuable. A clinical clostridial strain can be identified by clustering its microarray data with the reference strain as shown in Fig. 1. The microarray clustering data has been shown to be consistent to the pulse-field gel electrograms used for analysis of isolates within a species (Janvilisri et al., 2009). With the flexibility of array customization, reducing costs and more easy-to-use analysis tools, this method will certainly be an attractive diagnostic tool for *Clostridium* species and epidemiological studies of *Clostridium* infection.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This project was supported with Federal funds from the National Institute of Allergy and Infectious Diseases, National Institute of Health, Department of Health and Human Services under contract, N01-AI-30054, Project No. ZC005-06. We would like to thank Drs. D.N. Gerding at Hines Veterans Affairs Hospital, A. Dascal at McGill University, L.G. Arroyo at University of Guelph, J.G. Songer at University of Arizona, B.M. Limbago at Centers for Disease Control and Prevention, and S.B. Melville at Virginia Polytechnic Institute and State University for providing us *Clostridium* isolates. Thanks are extended to Dr. Sean McDonough for his critical reading of the manuscript.

References

- Asha NJ, Wilcox MH. Laboratory diagnosis of *Clostridium perfringens* antibiotic-associated diarrhoea. *J Med Microbiol* 2002;51:891–894. [PubMed: 12435070]
- Barrett T, Edgar R. Gene expression omnibus: microarray data storage, submission, retrieval, and analysis. *Methods Enzymol* 2006;411:352–369. [PubMed: 16939800]
- Bartlett JG, Gerding DN. Clinical recognition and diagnosis of *Clostridium difficile* infection. *Clin Infect Dis* 2008;46(Suppl 1):S12–18. [PubMed: 18177217]
- Bodrossy L, Sessitsch A. Oligonucleotide microarrays in microbial diagnostics. *Curr Opin Microbiol* 2004;7:245–254. [PubMed: 15196491]
- Brook I. Current concepts in the management of *Clostridium tetani* infection. *Expert Rev Anti Infect Ther* 2008;6:327–336. [PubMed: 18588497]
- Bruggemann H. Genomics of clostridial pathogens: implication of extrachromosomal elements in pathogenicity. *Curr Opin Microbiol* 2005;8:601–605. [PubMed: 16125440]
- Brynestad S, Granum PE. *Clostridium perfringens* and foodborne infections. *Int J Food Microbiol* 2002;74:195–202. [PubMed: 11981970]
- Carman RJ, Van Tassell RL, Wilkins TD. The normal intestinal microflora: ecology, variability and stability. *Vet Hum Toxicol* 1993;35(Suppl 1):11–14. [PubMed: 8236752]
- Delmee M, Van Broeck J, Simon A, Janssens M, Avesani V. Laboratory diagnosis of *Clostridium difficile*-associated diarrhoea: a plea for culture. *J Med Microbiol* 2005;54:187–191. [PubMed: 15673515]
- Dudoit S, Gentleman RC, Quackenbush J. Open source software for the analysis of microarray data. *BioTechniques* 2003;(Suppl):45–51. [PubMed: 12664684]

- Gerding DN, Muto CA, Owens RC Jr. Treatment of *Clostridium difficile* infection. Clin Infect Dis 2008;46(Suppl 1):S32–42. [PubMed: 18177219]
- Gurjar AA, Hegde NV, Love BC, Jayarao BM. Real-time multiplex PCR assay for rapid detection and toxotyping of *Clostridium perfringens* toxin producing strains in feces of dairy cattle. Mol Cell Probes 2008;22:90–95. [PubMed: 17890052]
- Hacia JG, Collins FS. Mutational analysis using oligonucleotide microarrays. J Med Genet 1999;36:730–736. [PubMed: 10528850]
- Hatheway CL. Toxigenic clostridia. Clin Microbiol Rev 1990;3:66–98. [PubMed: 2404569]
- Janvilisri T, Scaria J, Thompson AD, Nicholson A, Limbago BM, Arroyo LG, Songer JG, Gröhn YT, Chang YF. Microarray identification of *Clostridium difficile* core components and divergent regions associated with host origin. J Bacteriol 2009;191:3881–3891. [PubMed: 19376880]
- Lindstrom M, Keto R, Markkula A, Nevas M, Hielm S, Korkeala H. Multiplex PCR assay for detection and identification of *Clostridium botulinum* types A, B, E, and F in food and fecal material. Appl Environ Microbiol 2001;67:5694–5699. [PubMed: 11722924]
- Loy A, Bodrossy L. Highly parallel microbial diagnostics using oligonucleotide microarrays. Clin Chim Acta 2006;363:106–119. [PubMed: 16126187]
- Lyons P. Advances in spotted microarray resources for expression profiling. Brief Funct Genomic Proteomic 2003;2:21–30. [PubMed: 15239940]
- Mikhailovich V, Gryadunov D, Kolchinsky A, Makarov AA, Zasedatelev A. DNA microarrays in the clinic: infectious diseases. BioEssays 2008;30:673–682. [PubMed: 18536036]
- Palaniappan RU, Zhang Y, Chiu D, Torres A, Debroy C, Whittam TS, Chang YF. Differentiation of *Escherichia coli* pathotypes by oligonucleotide spotted array. J Clin Microbiol 2006;44:1495–1501. [PubMed: 16597882]
- Papoutsakis ET. Engineering solventogenic clostridia. Curr Opin Biotechnol 2008;19:420–429. [PubMed: 18760360]
- Persson S, Torpdahl M, Olsen KE. New multiplex PCR method for the detection of *Clostridium difficile* toxin A (tcdA) and toxin B (tcdB) and the binary toxin (cdtA/cdtB) genes applied to a Danish strain collection. Clin Microbiol Infect 2008;14:1057–1064. [PubMed: 19040478]
- Peterson LR, Kelly PJ, Nordbrock HA. Role of culture and toxin detection in laboratory testing for diagnosis of *Clostridium difficile*-associated diarrhea. Eur J Clin Microbiol Infect Dis 1996;15:330–336. [PubMed: 8781886]
- Scaria J, Chandramouli U, Verma SK. Antibiotic Resistance Genes Online (ARGO): a Database on vancomycin and beta-lactam resistance genes. Bioinformatics 2005;1:5–7. [PubMed: 17597841]
- Scaria J, Palaniappan RU, Chiu D, Phan JA, Ponnala L, McDonough P, Grohn YT, Porwollik S, McClelland M, Chiou CS, Chu C, Chang YF. Microarray for molecular typing of *Salmonella enterica* serovars. Mol Cell Probes 2008a;22:238–243. [PubMed: 18554865]
- Scaria J, Sreedharan A, Chang YF. Microbial Diagnostic Array Workstation (MDAW): a web server for diagnostic array data storage, sharing and analysis. Source Code Biol Med 2008b;3:14. [PubMed: 18811969]
- Snell H, Ramos M, Longo S, John M, Hussain Z. Performance of the TechLab C. DIFF CHEK-60 enzyme immunoassay (EIA) in combination with the C. difficile Tox A/B II EIA kit, the Triage C. difficile panel immunoassay, and a cytotoxin assay for diagnosis of *Clostridium difficile*-associated diarrhea. J Clin Microbiol 2004;42:4863–4865. [PubMed: 15472364]
- Sobel J. Botulism. Clin Infect Dis 2005;41:1167–1173. [PubMed: 16163636]
- Stabler RA, Gerding DN, Songer JG, Drudy D, Brazuer JS, Trinh HT, Witney AA, Hinds J, Wren BW. Comparative phylogenomics of *Clostridium difficile* reveals clade specificity and microevolution of hypervirulent strains. J Bacteriol 2006;188:7297–7305. [PubMed: 17015669]
- Turgeon DK, Novicki TJ, Quick J, Carlson L, Miller P, Ulness B, Cent A, Ashley R, Larson A, Coyle M, Limaye AP, Cookson BT, Fritsche TR. Six rapid tests for direct detection of *Clostridium difficile* and its toxins in fecal samples compared with the fibroblast cytotoxicity assay. J Clin Microbiol 2003;41:667–670. [PubMed: 12574264]
- Yang J, Chen L, Sun L, Yu J, Jin Q. VFDB 2008 release: an enhanced web-based resource for comparative pathogenomics. Nucleic Acids Res 2008;36:D539–542. [PubMed: 17984080]

Figure 1.

Hierarchical clustering of *Clostridium* 65 strains based on the microarray data. The strain codes are indicated. Two arrays for each strain were averaged and analyzed with MDAW. The diagram was arranged with the positive control probes for rRNA genes at the top, followed by probes specific to each *Clostridium* species indicated on the right. Each column represents an isolate and each row corresponds to a probe. The status of each probe is indicated by color as follows: red, present; green, absent; and gray, not tested for species-specificity (*C. boltae*, *C. botulinum*, and *C. leptum*) but no-cross specificity to other tested species.

Figure 2. Distribution of the known or putative virulence related genes among *Clostridium* isolates. Each row corresponds to a gene and each column in each panel represents a test strain. The status of each gene is indicated by color as follows: red, present/conserved; green, absent; gray, divergent. The designations of these genes are indicated on the right.

Table 1

Bacterial strains used in this study.

No.	Code	Strain No.	Strain	Source*
1	CB1	8489	<i>C. butyricum</i>	CDC
2	CB2	8490	<i>C. butyricum</i>	CDC
3	CB3	8491	<i>C. butyricum</i>	CDC
4	CB4	8492	<i>C. butyricum</i>	CDC
5	CB5	8493	<i>C. butyricum</i>	CDC
6	CB6	8494	<i>C. butyricum</i>	CDC
7	CB7	8495	<i>C. butyricum</i>	CDC
8	CB8	8496	<i>C. butyricum</i>	CDC
9	CB9	8497	<i>C. butyricum</i>	CDC
10	CC	8562	<i>C. chauvoei</i> 995	JGS
11	CD1	6372	<i>C. difficile</i> 630	DNG
12	CD2	6377	<i>C. difficile</i> 114	LGA
13	CD3	6381	<i>C. difficile</i> 124	LGA
14	CD4	6413	<i>C. difficile</i>	CDC
15	CD5	6425	<i>C. difficile</i>	CDC
16	CD6	6427	<i>C. difficile</i>	CDC
17	CD7	6442	<i>C. difficile</i>	CDC
18	CD8	6515	<i>C. difficile</i> 32g58	LGA
19	CD9	6519	<i>C. difficile</i> 651	LGA
20	CD10	6525	<i>C. difficile</i> 661	LGA
21	CG	8563	<i>C. glycolicum</i> 6031	JGS
22	CN	8508	<i>C. novyi</i> ATCC 19402	ATCC
23	CP1	8350	<i>C. perfringens</i> 13	SBM
24	CP2	8351	<i>C. perfringens</i> ATCC 13124	ATCC
25	CP3	8352	<i>C. perfringens</i> SM101	SBM
26	CP4	8451	<i>C. perfringens</i>	CDC
27	CP5	8452	<i>C. perfringens</i>	CDC
28	CP6	8453	<i>C. perfringens</i>	CDC
29	CP7	8454	<i>C. perfringens</i>	CDC
30	CP8	8455	<i>C. perfringens</i>	CDC
31	CP9	8456	<i>C. perfringens</i>	CDC
32	CP10	8457	<i>C. perfringens</i>	CDC
33	CSD1	8479	<i>C. sordelli</i>	CDC
34	CSD2	8480	<i>C. sordelli</i>	CDC
35	CSD3	8481	<i>C. sordelli</i>	CDC
36	CSD4	8482	<i>C. sordelli</i>	CDC
37	CSD5	8483	<i>C. sordelli</i>	CDC
38	CSD6	8484	<i>C. sordelli</i>	CDC
39	CSD7	8485	<i>C. sordelli</i>	CDC
40	CSD8	8486	<i>C. sordelli</i>	CDC

No.	Code	Strain No.	Strain	Source*
41	CSD9	8487	<i>C. sordelli</i>	CDC
42	CSG1	8459	<i>C. sporogenes</i>	CDC
43	CSG2	8460	<i>C. sporogenes</i>	CDC
44	CSG3	8461	<i>C. sporogenes</i>	CDC
45	CSG4	8462	<i>C. sporogenes</i>	CDC
46	CSG5	8463	<i>C. sporogenes</i>	CDC
47	CSG6	8464	<i>C. sporogenes</i>	CDC
48	CSG7	8465	<i>C. sporogenes</i>	CDC
49	CSG8	8466	<i>C. sporogenes</i>	CDC
50	CST1	8469	<i>C. septicum</i>	CDC
51	CST2	8470	<i>C. septicum</i>	CDC
52	CST3	8472	<i>C. septicum</i>	CDC
53	CST4	8473	<i>C. septicum</i>	CDC
54	CST5	8474	<i>C. septicum</i>	CDC
55	CST6	8476	<i>C. septicum</i>	CDC
56	CST7	8477	<i>C. septicum</i>	CDC
57	CST8	8478	<i>C. septicum</i>	CDC
58	CT1	8500	<i>C. tetani</i>	CDC
59	CT2	8501	<i>C. tetani</i>	CDC
60	CT3	8502	<i>C. tetani</i>	CDC
61	CT4	8503	<i>C. tetani</i>	CDC
62	CT5	8504	<i>C. tetani</i>	CDC
63	CT6	8505	<i>C. tetani</i>	CDC
64	CT7	8506	<i>C. tetani</i>	CDC
65	CT8	8507	<i>C. tetani</i>	CDC

* CDC = Center of Disease Control and Prevention, Atlanta, GA

JGS = J G Songer, Department of Veterinary Science and Microbiology, University of Arizona, Tucson, AZ

DNG = DN Gerding, Hines Veterans Affairs Hospital, Hines, IL

LGA= LG Arroyo, Department of Pathobiology, Ontario Veterinary College, University of Guelph, Guelph, ON, Canada

ATCC = The American Type Culture Collection, Manassas, VA

SBM = SB Melville, Department of Biological Sciences, Virginia Polytechnic Institute and State University, Blacksburg, VA