ICAAC
2014
D-200

The OpGen® *C. difficile* DNA Complete Test: a new qualitative molecular diagnostic test for the direct detection and virulence profiling of *Clostridium difficile*

T. Rockweiler, T. Wagner, D. Toal and T. Walker

OpGen, Inc., Gaithersburg, MD USA

OpGen In.
301-869-9683
twalker@opgen.com

ABSTRACT

Background:

Clostridium difficile is the most common infectious cause of diarrhea in hospitalized patients. *C. difficile* can express three major toxins: toxin A (*tcd*A), toxin B (*tcd*B) and binary toxin (*cdt*B). *C. difficile type* NAP1/027 has caused epidemics in North America and Europe due to mutation in a fourth toxin gene, toxin C (*tcd*C.)[1] This study assessed sensitivity, specificity, reproducibility and accuracy for a new molecular diagnostic test that provides a more complete profile of toxigenic *C. difficile* through detection of genes for toxin A, toxin B, binary toxin and the NAP1 mutation.

Methods:

The *C. difficile* DNA Complete Test (OpGen, Gaithersburg, MD) is a PCR-based *in vitro* diagnostic for direct detection of gastrointestinal colonization with toxigenic *C. difficile* . The assay screens and differentially reports three *C. difficile* genes encoding toxins (*tcd*A, *tcd*B, and *cdt*B) and the NAP1 mutation. Analytical sensitivity, specificity, and reproducibility were tested by spiking a pool of several negative stool specimens with *C. difficile* isolates with various toxigenic genotypes. Accuracy of the *toxin B* assay was assessed using 40 stool specimens spiked with four levels of *C. difficile* ATCCC BAA-1870. Samples were blinded and split for comparison testing with the *C. difficile* DNA Complete Test at OpGen and an FDA approved reference method from a second laboratory.

Results:

Limit of detection for the OpGen *C.difficile* DNA Complete Test was 480 colony forming units per swab for all four toxin genes. The assay was reproducible over multiple batches and operators without cross-reactivity with other bacteria found in stool. Using the *C. difficile* DNA Complete Test, specimens at high, medium, low and negative levels results were 100% concordant. The reference laboratory results at high, medium, low, and negative levels results were 100%, 100%, 10%, and 100%, respectively.

Conclusion:

The OpGen *C. difficile* DNA Complete Test provides sensitive, specific and accurate detection and virulence profiling of toxigenic *C. difficile* in stool samples. The assay identifies the hypervirulent NAP1/027 strain by detecting the toxin C mutation associated with over-expression of toxin A and toxin B

MATERIALS & METHODS

Specimen Preparation

Stool samples for this study were prepared using a pool of stool from individual donors sourced from Discovery Life Sciences (Los Osos, CA) The pool was subsequently spiked with the organisms of interest at varying amounts. (Refer to each section for defined organisms and levels.) Specimens were prepared for analysis utilizing a modified Qiagen DNA Stool Mini Kit (Qiagen, 51504) to extract nucleic acids from dual swab dipped in stool.

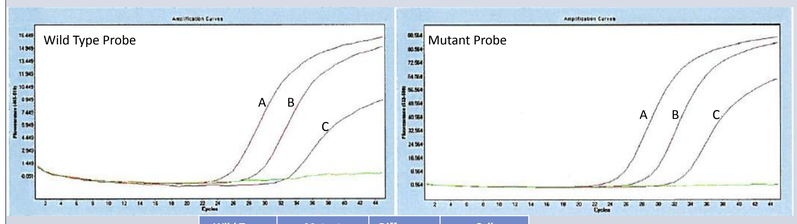
PCR

Specimens were analysis utilizing real-time PCR on the Roche LightCycler® 480 II System, a 96-well plate-based platform.

Toxin C Analysis

PCR for the detection of toxin C utilized two hydrolysis probes; one specific for the wild type strains and one specific for the mutant NAP1/027 deletion. Each of the probes in labeled with a different fluorophore with the LightCycler® set to monitor the wavelengths for each of the fluorophores during PCR. The toxin C data from both traces are analyzed using 'Fit Points' with the NoiseBand manual fluorescence baseline set to 3.000. Samples results are determined by taking the Cp (Crossing Point) value of the mutant result minus wild type result. If the value is greater than zero, then the sample is wild type. If the value is less than zero, the sample is mutant.

Example of toxin C mutant strain.



Wild Type		Mutant		Difference	Call
	Cp		CP		
A	26.78	A	23.98	-2.8	Mutant
B	30.28	B	27.58	-2.7	Mutant
C	33.65	C	31.9	-1.75	Mutant

A= Oxford 1407
B= Oxford 1407 1:10
C=Oxford 1407 1:100

RESULTS

Analytical Sensitivity

Analytical sensitivity (limit of detection) for each target gene assay was demonstrated using negative stool that was quantitatively spiked with bacterial culture isolate harboring four toxin genes (ATCC-BAA-1870.) Spiked culture levels were determined by parallel counting of colony forming units (CFUs) on culture plates.

C. difficile DNA Complete Test as developed at OpGen, Inc. has a limit of detection of 480 CFU/dual swab for all 4 gene targets. This represents the lowest number of CFU/dual swab at which a positive result for any of the 4 gene targets will be obtained with at least 95% confidence.

Specificity (Toxin C genotypes)

In addition to testing assay specificity against common GI organisms, the *C. difficile* Complete Test was tested to show its ability to differentiate the 4 common genotypes for toxin C at nucleotide 117. 4 different Oxford strains representing the 4 common genotypes were tested at three 10-fold dilutions levels of input genomic DNA. Results for toxin C genotypes and the remaining three target genes (toxin A, toxin B, and binary) were compared with Oxford pubMLST database.

	PubMLST Result from Oxford University					C. difficile Complete Test			
Oxford Strain	Toxin A	Toxin B	Toxin C Genotype at Nucleotide Position 117	Binary Toxin		Toxin A	Toxin B	Toxin C Genotype at Nucleotide Position 117	Binary Toxin
1407	+	+	NAP1 deletion	+		+	+	NAP1 deletion	+
1518	+	+	T	+		+	+	T	+
110	+	+	G	-		+	+	G	-
1396	+	+	A	-		+	+	A	-

All 4 genotype calls for toxin C were accurate at all dilutions levels for the tested isolates. Results also matched 100% with the pubMLST database for toxin C and for the remaining toxins (toxin A, toxin B, and binary).

Specificity

Specificity was tested for each of the 4 gene targets against most commonly found bacteria in the GI tract. 13 organisms were tested at purified DNA levels equivalent to 10^6 to 10^8 genomic copies per PCR. The test is specific to toxigenic *C. difficile* for all 4 targets that encompass the panel.

Species	Strain	Toxin A	Toxin B	Binary	Toxin C deletion
Bacteriodes fragilis	ATCC 29771	Negative	Negative	Negative	Negative
Enerococcus faecalis	ATCC 49332	Negative	Negative	Negative	Negative
Enterococcus faecium	ATCC 51858	Negative	Negative	Negative	Negative
Citrobacter freundii	ATCC 33128	Negative	Negative	Negative	Negative
Proteus mirabilis	ATCC 12453	Negative	Negative	Negative	Negative
Salmonella enterica	CCUG 49323	Negative	Negative	Negative	Negative
Serratia marcescens	ATCC 43862	Negative	Negative	Negative	Negative
Shigella sonei	Washington State	Negative	Negative	Negative	Negative
Staphylococcus aureus	USA 800 CDC 189	Negative	Negative	Negative	Negative
Clostridium bifermentans	CCUG 32113	Negative	Negative	Negative	Negative
Clostridium difficile (non-toxic)	ATCC 70057	Negative	Negative	Negative	Negative
Clostridium septicum	CCUG 46934	Negative	Negative	Negative	Negative
Clostridium tetani	CCUG 52330	Negative	Negative	Negative	Negative

RESULTS (cont.)

Accuracy

Accuracy was determined by testing 40 stool specimens that were spiked with varying quantities of *C. difficile* harboring all 4 targets genes.

Sample	Replicates
High (Positive)	10
Med (Positive)	10
Low (Positive)	10
Negative	10

These 40 specimens were blinded and split to be tested at OpGen and at a Quest Diagnostics (BD MAX™ Cdiff Assay) for the toxin B target. The remaining 3 targets were also tested at OpGen labs and study results compared against expected results based on spiking known quantities of *C. difficile*.

Percent Agreement to Expected Results-Toxin B				
	(High) Positives	(Med) Positives	(Low) Positives	Negatives
C. difficile Complete Test Result	100%	100%	100%	100%
BD MAX™ Cdiff Assay Result	100%	100%	10%	100%

Percent Agreement to Expected Results- Toxin A, Toxin C, and Binary				
	(High) Positives	(Med) Positives	(Low) Positives	Negatives
C. difficile Complete Test Result	100%	100%	100%	100%

Reproducibility

A panel of specimens with varying concentrations of *C. difficile* were tested for reproducibility. Based on the estimated limit of detection to be at 480 CFU/dual swab, three different samples were generated by spiking in *C. difficile* isolate harboring the 4 target genes in to negative stool specimen at 10, 100, and 1000 fold above the limit of detection. Three extractions were performed per concentration of samples on 3 different days

	Day One			Day Two			Day Two		
Target	1000x Target Level (Cp)	100x Target Level (Cp)	10x Target Level (Cp)	1000x Target Level (Cp)	100x Target Level (Cp)	10x Target Level (Cp)	1000x Target Level (Cp)	100x Target Level (Cp)	10x Target Level (Cp)
Toxin A	31.88	34.85	38.51	29.43	31.63	33.36	28.68	32.52	37
Toxin B	31.75	34.83	38.39	29.66	31.8	33.4	28.72	32.35	36.68
Toxin C deletion	31.63	33.75	35.04	29.22	31.03	32.04	28.76	31.65	34.21
Binary	31.38	34.37	37.62	29.32	31.52	33.14	28.59	32.26	36.58

CONCLUSIONS

1. *C. difficile* DNA Complete Test provides high sensitive and specific detection of toxigenic *C. difficile* in stool specimens.
2. *C. difficile* DNA Complete Test provides superior sensitivity compared to BD MAX™ Cdiff Assay.
3. *C. difficile* DNA Complete Test accurately differentiates toxin C genotypes properly identifying hypervirulent strain NAP1/027.

REFERENCES

[1] Guide to Preventing *Clostridium difficile* infections. APIC 2013

[2] **de Boer, R. F. et al.** 2010. Evaluation of a rapid molecular screening approach for the detection of toxigenic *Clostridium difficile* in general and subsequent identification of the tcdC Delta 117 mutation in human stools. J. Microbiol Methods 83:59-65

[3] **Wroblewski, D., Hannett, G. E. Bopp, D. J., Dumyati, G. K., Halse, T. A., Dumas, N. B., & Musser, K. A.** (2009) Rapid Molecular Characterization of *Clostridium difficile* and Assessment of Populations of *C. difficile* in Stool Specimens. Journal of Clinical Microbiology 47(7), 2142-2148