Multi-drug resistant *Clostridium difficile* ribotype 027 in southwestern Virginia, 2007-2013: Common, fit and virulent

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ABSTRACT

Background: Ribotype 027 is a strain of *C. difficile* that emerged about 15 years ago. Today's 027 isolates are almost always fluoroquinolone resistant. Some consider 027 highly virulent, causing worse symptoms and outcomes. Others disagree. **Material/methods:** We assayed sequential culture positive, anonymous and unlinked stool samples and their recovered isolates. **Results:** Between 2007 and 2013, 027 was the most common of 128 *C. difficil*e ribotypes in southwestern Virginia. 027 isolates were 32% of 3118 isolates. >98% of 027 isolates were fluoroquinolone resistant (FQR). The majority (>75%) of 027FQR was also resistant to erythromycin, clindamycin and rifampicin. 027FQR was in 45% of cytotoxin positive but only 17% of cytotoxin negative fecal samples (p≤0.05) and 34% of unformed but only 21% of formed stool samples (p≤0.05). The association of 027FQR with unformed stool and toxins and the reverse for 014/020 and non-toxigenic isolates suggested 027FQR is virulent and 014/020 and the non-toxigenic isolates are not. Samples from a subset of patients infected with 027FQR had higher counts, higher concentrations of toxins and more lactoferrin (n=15, $10^{5.2}$ cfu/g feces, 157 ng TcdA/g, 180 ng TcdB /g, 163 ng CdtB/g, 410 µg/g lactoferrin) than those infected with 014/020 (n=9, $10^{3.5}$ cfu/g, 11 ng/g*, 1 ng/g*, 0 ng/g* and 39 µg/g* respectively*p>0.05 vs 027) and non-toxigenic isolate (n=17, 103.7 cfu/g, 0 ng/g*, 0 ng/g*, 0 ng/g*, 42 µg/g* respectively). **Conclusions:** Ribotype contributed to virulence, affecting both bioburden, toxin levels, and host response.

BACKGROUND

Recent reports stress the significance of fecal toxin to the severity of *C. difficile* diarrhea. The absence of toxin is associated with less severe presentations, the presence of fecal toxin with worse. Furthermore, there are indications that the presence of fecal toxin is linked to higher bioburdens and vice versa. Overlapping with these trends is the possible role of ribotype. *C. difficile* ribotype 027 has been linked to the presence of fecal toxin and severity of *C. difficile* diarrhea, suggesting that 027 may be a more virulent ribotype. We tested this possibility by assaying fecal samples and the isolates recovered from them, focusing on markers of colonization and fitness, virulence and host inflammatory response.

MATERIALS AND METHODS

Stool specimens: We used already existing, anonymous and unlinked fecal samples submitted to single southwestern Virginia clinical laboratory for routine *C. difficile* testing. The Bristol Stool Chart was used to report consistency.

Glutamate dehydrogenase (GDH): GDH was assayed by the TECHLAB C. DIFF CHEK ™ -60 test according to the Package Insert and, quantitatively (ng/mL), using a modified TECHLAB C. DIFF CHEK™-60.

Toxins: These were quantified using purified toxin standards and individual toxin-specific modifications of TECHLAB's C. DIFFICILE TOX A/B II™ test. Toxin B was measured with the TECHLAB C. DIFFICILE TOX-B TEST and results were reported as Yes/No. Binary toxin binding component was assayed by an ELISA.

Lactoferrin: Lactoferrin was measured using the TECHLAB LACTOFERRIN SCAN.

Bacterial counts: We counted CFU/g feces on CCFA.

Antibiotic resistances: Resistance was established by Etest.

PCR analysis and PCR ribotyping: DNA was extracted from broth cultures using the QIAamp Mini Kit (Qiagen, Valencia, CA) and amplified. Banding patterns were compared to TECHLAB's ribotype library. In addition we grouped ribotypes into four divisions (below) based on additional PCR testing for *tcdA, tcdB* and *cdtB* that were confirmed by toxin-specific immunoassays.

TABLE 1. FLUOROQUINOLONE RESISTANT *C. DIFFICILE* 027 IN SOUTHWESTERN VIRGINIA - MULTIDRUG RESISTANT AND COMMON

Rank	Ribotype[1]	Toxin phenotype[2]	% of 3118	% Resistant[3]			
				MOX	ERM	CLIND	RIF
1	027 FQR	ABC	31.6	100	96	76	75
2	053 FQR	AB	9.7	100	99	99	4
3	014/020	AB	9.5	13	16	9	2
4	039	Nontoxigenic	3.8	64	85	87	1
5	010	Nontoxigenic	3.7	11	37	35	2
6	106	AB	3.6	9	8	8	0
7	009	Nontoxigenic	3.4	9	16	12	2
8	002	AB	3.3	3	4	4	1
9	056	AB	2.8	3	10	5	2
10	126	ABC	2.3	10	20	20	7
11	001	AB	2.0	42	7	7	0
12	054	AB	1.5	9	3	0	0
13	005	AB	1.5	4	16	16	0
14	015	AB	1.5	7	6	25	0
15	017	B	1.3	28	80	33	5
16	057	AB	1.2	12	0	4	0
17	032	Nontoxigenic	1.0	7	13	4	0
18	012	AB	1.0	3	76	68	0
19	027 FQS	ABC	0.9	0	7	7	0
20	046	AB	0.9	23	42	42	0
31	244	ABC	0.4	0	0	0	0
40	053 FQS	AB	0.2	0	0	0	0
59	078	ABC	0.1	0	ND	ND	ND
	Other toxigenic ribotypes[4]		9.7	NA	NA	NA	NA
	Other nontoxigenic ribotypes[5]		3.3	NA	NA	NA	NA

[1] FQS = Fluoroquinolone sensitive; FQR= Fluoroquinolone resistant
[2] A = TcdA, B = TcdB, C = Binary toxin (CDT)
[3] MOX = moxifloxacin, ERM = erythromycin, CLIND = clindamycin, RIF = rifampicin
[4] Other toxigenic ribotypes were: 003, 004, 006, 011, 013, 018, 020, 023, 024, 029, 034, , 043, 050, 061, 066, 072, 075, 080, 081, 096, 097, 101, 103 104, 107, 109, 116, 118, 131, 137, 147, 153, 154, 158, 159, 173, 180, 198, 208, 216, 220, 245, 248, 251, 274, 284, 293, 305, 334, 355, 378, 379, 386, 389, 394, 400, 401, 403, 404, 408, 417, 462, 469, , 476, 521, 523, 524, UNK 7 UNK 8, UNK 23, UNK 24, UNK 28, UNK 29, and UNK 30. (UNK = unknown)
[5] Other nontoxigenic ribotypes were: 031, 035, 037, 038, 051, 071, 073, 082, 085, 088, 115, 150, 155, 184, 188, 307, 328, 396, 397, 399, 405, 406, 409, 410, 420, 450, 474, 480, and UNK 27

- 027FQR - 32% of all isolates, ranked 1st .
- 75% of 027FQR isolates were also resistant to erythromycin, clindamycin and rifampicin.
- 027FQS - 1% of all isolates, ranked 19th.
- 7 % of 027FQS isolates were also resistant to erythromycin and clindamycin; none was resistant to rifampicin.
- The same trends were seen for 053FQR and 053FQS, ranked 2nd and 40th respectively.

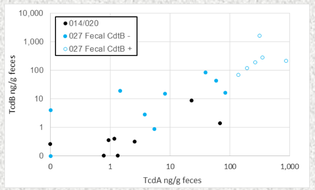

FIG 1. *IN VIVO* TOXIN AND RIBOTYPE

TcdA and TcdB ~equimolar.

CdtB was present only at high TcdA and TcdB levels

027FQR achieved high toxin levels, 014/020 did not

FIG 2. *IN VIVO* TOXIN AND COUNT

TcdB level rose with count

High TcdB levels with 027FQR
Low TcdB with 014/020 samples

CdtB at highest TcdB levels, not highest counts

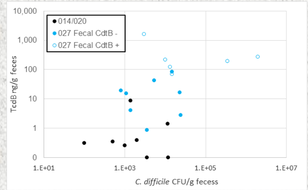

TABLE 2. FLUOROQUINOLONE RESISTANT *C. DIFFICILE* 027: ASSOCIATION WITH FECAL TOXIN AND STOOL CONSISTENCY = VIRULENT

Ribotype[1]	Toxin phenotype[2]	% frequency in samples		Fisher Exact p value	Significant association
		Non-cytotoxin (n=1484)	Cytotoxic (n=1634)		
027FQR	ABC	17	45	0.0001	Associated with fecal cytotoxin
053FQR	AB	7	12	0.001	
244	ABC	0.1	0.4	0.002	
027 FQS	ABC	0.7	1	0.3	No association
053 FQS	AB	0.4	None	0.02	Associated with absence of fecal cytotoxin
031	Nontoxigenic	0.7	0.1[3]	0.01	
002	AB	4	3	0.01	
014/020	AB	11	8	0.01	
038	Nontoxigenic	0.7	0.1[3]	0.005	
073	Nontoxigenic	0.8	0.1[3]	0.001	
010	Nontoxigenic	7	0.5[3]	0.001	
009	Nontoxigenic	7	0.2[3]	0.001	
039	Nontoxigenic	7	0.5[3]	0.0001	
032	Nontoxigenic	2	0.1[3]	0.0001	
085	Nontoxigenic	1	0.1[3]	0.0001	
Ribotype[1]	Toxin phenotype[2]	% frequency in samples		Fisher Exact p value	Significant association
		Formed (n=508)	Unformed (n=2610)		
027 FQR	ABC	21	34	0.00001	Associated with unformed stool
053FQR	AB	8	9	0.6	No association
027 FQS	ABC	0.6	0.9	0.8	
017	B	2	1	0.05	Associated with formed stool
014/020	AB	14	9	0.002	
002	AB	6	3	0.003	
053 FQS	AB	2	0.2	0.00004	

[1] Only ribotypes occurring at ~1% or more of all isolates are shown; FQR = Fluoroquinolone resistant; FQS = Fluoroquinolone sensitive
[2] A = TcdA, B = TcdB, C = Binary toxin (CDT)
[3] A few samples were found in which cytotoxicity was present but only a toxigenic isolate was recovered. Coinfections are well documented.

- 027FQR associated with fecal toxin and unformed stools – cause and symptom of *C. difficile* diarrhea - 027FQR is virulent.
- 027FQS is not associated with toxin or diarrhea – lower virulence than 027FQR or FQ use by infected patients selects for 027FQR.
- Same trends were seen for 053FQR and 053FQS
- 014/020 and nontoxigenic ribotypes were associated with the absence of toxin and with formed stools - 014/020 is less virulent than 027

TABLE 3. FLUOROQUINOLONE RESISTANT *C. DIFFICILE* 027: HIGH MEAN BIOBURDEN, 3 TOXINS, AND HIGH TOXIN/G FECES AND TOXIN/CFU = FIT AND VIRULENT

Ribotype	*C. difficile* CFU/g feces	TcdA ng/g feces	TcdB ng/g feces	CdtB ng/g feces[1]	TcdA pg/CFU	TcdB pg/CFU	CdtB ng/CFU	Lactoferrin µg/g feces
027FQR (n=15)	$10^{5.2}$	157	180	163	16	4	16	410
014/020FQS (n=9)	$10^{3.6}$	12*	1*	Not detected	6	1	Not detected	43**
Nontoxigenic (n=17)	$10^{3.7}$	Not detected	Not detected	Not detected	Not applicable	Not applicable	Not detected	33**

- ps0.1 and ** ps0.01 versus 027FQR. No other differences (ps0.1) were seen
[1] Only 6 of 15 samples contained recoverable CdtB

027FQR - 3rd toxin may enhance virulence

027FQR - high mean bioburden indicates high *in vivo* fitness

027FQR - high TcdA and TcdB/g feces, high TcdA and TcdB/CFU indicate virulence

027FQR - high lactoferrin/g feces – host response implies virulence

014/020 – bioburdens same as nontoxigenic ribotypes – less fit than 027FQR

014/020 fewer toxins, less toxin/g and less toxin/CFU – less virulent than 027FQR

014/020 – Less lactoferrin - host response confirms lower virulence

CONCLUSIONS

- Multidrug resistant 027FQR was common, fit, and virulent.
- 027FQR was associated with unformed samples and those containing toxins.
- 027FQR reached high mean bioburdens in feces and made high mean levels of TcdA/g feces and TcdB/g feces.
- TcdB reached particularly high levels in samples containing 027FQR.
- 027FQR produced high fecal levels of TcdA/CFU and TcdB/CFU.
- CdtB was seen only in fecal samples that had very high levels of TcdA and TcdB.
- 027FQR patients had high levels of fecal lactoferrin.
- 014/020 was common but showed less *in vivo* fitness and virulence. Host inflammation levels were similar to those in patients colonized with nontoxigenic isolates.
- Both ribotype and bioburden were factors in virulence.

LIMITATIONS

All samples were tested. None was discarded because it was a repeat, or from an already treated individual, or because the stool was formed.

~16% of samples were formed. We grew isolates from all of these and saw toxin in many, making it unlikely patients were already receiving treatment. It is possible instead that, with time, less severe *C. difficile* infections in adults resolved without antimicrobial treatment.

Our samples therefore were almost certainly unrepresentative of the same single time point or stage in the course of the patients' infections.

A better study would require standardized patients (biome, immune status, comorbidity and so on), the same regimen of predisposing antibiotic and the samples to be collected on the same day or stage in course of infection (day of onset, day of worst symptoms, etc.).

Without such studies, biological trends that stratify ribotypes, may not be statistically significant because of small samples numbers amplified by sample heterogeneity.

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