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Clostridioides (Clostridium) difficile (including epidemiology)

Characterization of *Clostridium difficile* isolates collected during a phase 2b clinical study with SYN-004 (ribaxamase) for the prevention of *C. difficile* infection

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ABSTRACT

During a Phase 2b study with SYN-004 (ribaxamase) for prevention of *Clostridium difficile* infection (CDI) conducted in North America and Eastern Europe, 45 C. *difficile* isolates from subjects with laboratory-confirmed CDI and or colonized with *C. difficile* were collected and characterized. Several *C. difficile* PCR ribotypes, including 027 and 198, were identified.

Clostridium difficile is the leading cause of hospital-acquired infections [1]. A significant risk factor for development of *C. difficile* infection (CDI) is the use of antibiotics, including intravenous (IV) β-lactam antibiotics (penicillins and cephalosporins) [2,3]. SYN-004 (ribaxamase) is an orally-administered β-lactamase intended for coadministration with IV β-lactam antibiotics [4,5]. The active enzyme is released in the upper GI tract where it is available to degrade β-lactam antibiotics excreted into the intestine. This is expected to protect the integrity of the gut microbiome and prevent opportunistic infections like CDI.

A Phase 2b study was performed in US, Canada, Bulgaria, Hungary, Poland, Romania and Serbia to determine whether co-administration of ribaxamase with ceftriaxone in subjects being treated for a lower respiratory tract infection would reduce the incidence of CDI as well as new colonization with *C. difficile* as compared with placebo (published abstract [6], clinitrials.gov NCT02563106). *C. difficile* isolates were collected from subjects who met the endpoint of CDI (three loose or watery stools in 24 h and a positive CDI lab test) or were colonized with *C. difficile* as determined by microbiologic culture. Fecal samples for colonization were collected at screening and at 72 h and 4-weeks post-antibiotics.

The ribotype of the *C. difficile* isolates was determined by PCR as previously reported [7]. Briefly, nucleic acid template DNA was prepared and mixed with the various primer sets for amplification of 16S-23S rRNA by PCR, and the products were then separated by gel electrophoresis. The ribotypes were determined by comparison against a TechLab, Inc. database of known ribotypes.

Forty-five *C. difficile* isolates were analyzed including, nine samples from CDI-positive subjects (8 in placebo and 1 in the ribaxamase group), one from a diarrheal sample that was *C. difficile* toxin-negative by assay, but grew *C. difficile*, and 35 C. *difficile* colonization samples from 28 subjects. Table 1 shows the results of the analysis of these samples. Forty-four of the frozen stocks grew *C. difficile*, but one (a screening colonization sample) failed to grow bacteria. Of the 44 samples which grew *C. difficile*, eight isolates were non-toxigenic RTs 031, 328, 474 or a previously unidentified RT designated as TechLab (TL) unknown 36 and were all from colonization samples. The other 36 isolates were toxigenic and from both diarrheal and colonization samples and included RTs 001, 005, 011, 014, 046, and 081 carrying Toxins A and B, as well as RTs 027 and 198 which also carry the *Clostridium difficile* transferase (CDT) [8,9]. RT 198 is an emerging ribotype originally identified in Hungary [10], related to RT 027 [11]

Of the ten *C. difficile* isolates collected from diarrheal samples, nine came from subjects who met the study endpoint of CDI, while one came from a placebo subject for which no toxin was detected in

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Table 1
Detailed analysis of the *C. difficile* isolates.

SID# [a, b]	Sample type	Treatment	Macro	Ribotype	Toxins [c]
303–007	72 h Colonization	SYN-004	N	014	A/B
303–008	72 h Colonization	PBO	N	081	A/B
303–011	**Diarrhea (CDI)**	PBO	Y	001	A/B
303–011	72 h Colonization [d]	PBO	Y	001	A/B
303–020	**Diarrhea (CDI)**	PBO	Y	027	A/B CDT
305–001	72 h Colonization	SYN-004	Y	046	A/B
305–001	4-Week Colonization	SYN-004	Y	046	A/B
306–002	72 h Colonization	SYN-004	Y	001	A/B
308–003	**Diarrhea (CDI)**	PBO	Y	027	A/B CDT
308–010	**Diarrhea** [e]	PBO	N	001	A/B
308–010	4-Week Colonization [f]	PBO	N	001	A/B
308–018	72 h Colonization	PBO	Y	027	A/B CDT
310–001	Screening Colonization	PBO	Y	011	A/B
311–012	72 h Colonization	PBO	N	027	A/B CDT
311–015	**Diarrhea (CDI)**	PBO	N	027	A/B CDT
311–017	**Diarrhea (CDI)**	PBO	N	027	A/B CDT
501–001	72 h Colonization	PBO	N	027	A/B CDT
503–002	**Diarrhea (CDI)**	PBO	N	014	A/B
503–002	Early Term Colonization [g]	PBO	N	001	A/B
505–003	Screening Colonization	PBO	N	031	Non-toxigenic
505–003	72 h Colonization [h]	PBO	N	005	A/B
505–005	**Diarrhea (CDI)**	SYN-004	N	027	A/B CDT
506–002	72 h Colonization	PBO	N	027	A/B CDT
506–002	4-Week Colonization	PBO	N	027	A/B CDT
507–002	72 h Colonization	PBO	N	328	Non-toxigenic
508–001	72 h Colonization	SYN-004	Y	198	A/B CDT
508–002	**Diarrhea (CDI)**	PBO	Y	198	A/B CDT
508–002	Early Term. Colonization [i]	PBO	Y	198	A/B CDT
509–003	4-Week Colonization	SYN-004	N	001	A/B
509–004	Screening Colonization	SYN-004	N	001	A/B
509–010	4-Week Colonization	SYN-004	N	014	A/B
509–014	Early Term. Colonization	SYN-004	N	474	Non-toxigenic
601–007	Screening Colonization	PBO	N	010	A/B
601–008	Screening Colonization	PBO	N	005	A/B
605–011	Screening Colonization	PBO	N	TL UNK 36	Non-toxigenic
605–011	72 h Colonization [j]	PBO	N	TL UNK 36	Non-toxigenic
605–013	4-Week Colonization	SYN-004	N	001	A/B
608–001	Screening Colonization	SYN-004	N	014	A/B
702–002	72 h Colonization	PBO	N	014	A/B
703–002	72 h Colonization	PBO	N	474	Non-toxigenic
703–002	4-Week Colonization	PBO	N	474	Non-toxigenic
720–004	Screening Colonization	SYN-004	Y	N/A [k]	N/A
722–002	**Diarrhea (CDI)**	PBO	Y	027	A/B CDT
722–002	Early Term. Colonization [l]	PBO	Y	027	A/B CDT
803–002	72 h Colonization	PBO	N	474	Non-toxigenic

The bold indicates the samples collected from diarrhea episodes.
[a] Abbreviations: SID#-subject identification number, PBO-placebo, Macro-macrolide administered, Y-yes, N-no, CDI-*C. difficile* infection, TL-TechLab, UNK-unknown, N/A-not applicable, Term-termination.
[b] 300 Serbia, 500 Romania, 600 Bulgaria, 700 Poland, 800 United States.
[c] *C. difficile* toxin A, *C. difficile* toxin B, binary toxin (CDT).
[d] Sample collected 10 days after positive diagnosis for CDI.
[e] Sample GDH positive, but no toxin detected in feces at the local or central lab.
[f] Sample collected 17 days after diarrheal sample.
[g] Separate early termination colonization sample (rectal swab) collected on the same day as CDI sample.
[h] Sample collected 11 days after screening sample.
[i] Sample collected 7 days after positive diagnosis for CDI.
[j] Sample collected 7 days after screening sample.
[k] No bacteria grew from frozen stock.
[l] Sample collected the next day after positive diagnosis for CDI.

the stool by either the local or the central laboratory. This sample was positive for glutamate dehydrogenase [12] and was culture positive for toxigenic RT 001. Six of the diarrheal isolates from CDI were RT 027, while one each was RT 198, 001 or 014. In terms of treatment assignment and ribotypes, the eight *C. difficile* isolates from placebo-treated subjects with CDI included five RT 027 and one RT 198, while one RT 027 isolate came from a CDI-positive ribaxamase subject.

RTs 027 and 198 were only found in the three study countries (Serbia, Romania and Poland) with laboratory-confirmed cases of CDI and accounted for seven of the nine cases. Six diarrheal isolates came from Serbia (five CDI and one toxin-negative diarrheal sample, four were RT 027), three CDIs from Romania (one was RT 027) and one CDI from Poland (RT 027). In Bulgaria, where over 100 subjects were enrolled in the study (Fig. 1), no laboratory-confirmed CDI were detected, and very little new colonization was detected. Even though Bulgaria did have one of the highest *C. difficile* colonization rates upon screening (3.9%), none of these isolates were RT 027 or 198. In Serbia, however, where there were four laboratory confirmed cases of CDI and eight cases of new colonization with *C. difficile*, RT 027 accounted for five of sixteen isolates. RT 027 and 198 were only found in four of the 31 samples

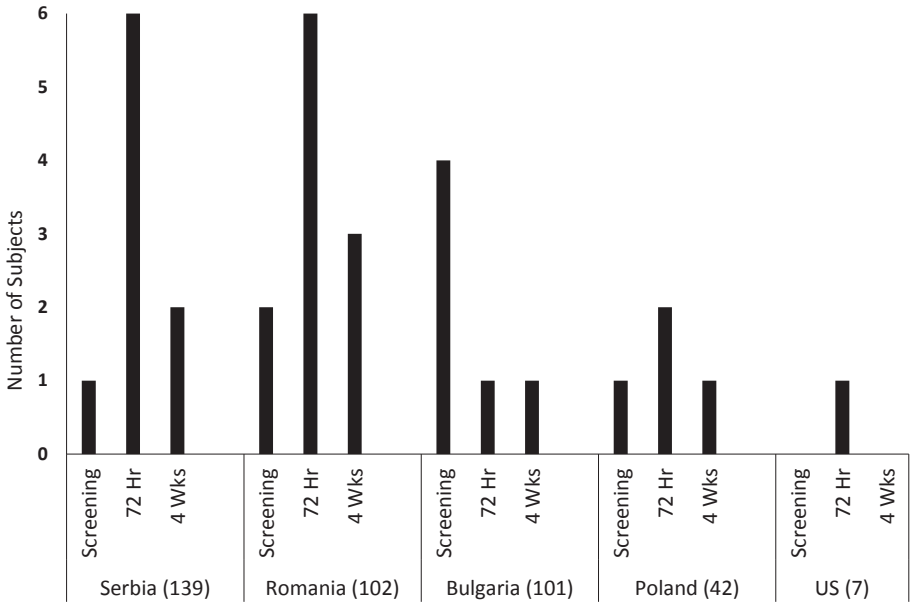

Fig. 1. C. *difficile* Colonization by Collection Point and Country. The graph displays the number of subjects who were colonized with *C. difficile* at each of the three collection points (screening, 72 h and 4 weeks post antibiotics) in the five participating countries which had colonized subjects. The numbers after the country names indicate the number of subjects enrolled in the study from that country. Some data represent a single subject who was colonized at two collection points.

from colonized subjects. These findings are consistent with previous reports that CDT-positive *C. difficile* isolates tend to be more virulent and more likely to cause disease than the CDT-negative strains [13,14]. RT 001 was also isolated from a confirmed CDI subject and from the subject with diarrhea but for which no toxin was detected (both from Serbia). This ribotype has been associated with severe infection in the nearby Czech Republic [15].

At the site level, there were three sites in Serbia (303 and 311, and 308) which had multiple cases of CDI during the study. Sites 303 and 311 each had two cases of CDI and two and one new *C. difficile* colonizations, respectively. Site 308 had a confirmed case of CDI as well as the case of diarrhea that was negative for detection of toxins yet yielded a toxigenic isolate of *C. difficile* upon culture and also had a new *C. difficile* colonization. At site 311, these isolates were all RT 027, but at the other two sites these were a mix of RT 027 and other toxigenic ribotypes.

For three of the subjects that met the study endpoint of CDI (303-011, 508-002 and 722-002), a colonization sample was also collected after their diagnosis, and for all three subjects, these samples were the same RT of *C. difficile* as found in their diarrheal sample. A fourth CDI subject (503-002), however, had RT 014 isolated from a diarrheal sample but a subsequent colonization sample collected on the same day yielded RT 001. The one diarrheal subject (308-010), who was *C. difficile* positive but toxin negative, had RT 001 in both the diarrheal sample and in a 4 week post-antibiotic sample collected 17 days after their diarrheal sample was collected.

During the study, subjects could also receive a macrolide as additional treatment and this appeared to be a site-specific decision. In five of the nine (56%) laboratory-confirmed cases of CDI, the subjects received a macrolide in addition to their ceftriaxone treatments, and in three of these five cases, the infecting strain was RT 027. This was compared with an overall rate of 36% for macrolide use during the study. This observation is consistent with previous reports which have associated an increased risk for CDI with multiple antibiotic use [2] and macrolide use has been associated with epidemic *C. difficile* strains [16]. These five cases of CDI were all in placebo-treated subjects which suggested that even though ribaxamase degrades only β-lactam antibiotics [4], it may protect against CDI in patients being treated with a β-lactam plus another antibiotic.

During the clinical study, new colonization was defined as a negative screening sample and then positive on one of two subsequent samples (72 h or 4 weeks post-antibiotics). Colonization with *C. difficile* varied by country (Fig. 1) with Romania and Serbia having the highest rates of new colonization (nine of 102 and eight of 139 subjects, respectively). Bulgaria had the highest rate of colonization on screening (four of 101 subjects), but Bulgaria only had two detectable new colonization incidents. In terms of *C. difficile* ribotypes found in the colonization samples from subjects who did not meet the endpoint of CDI, a mix of 13 ribotypes including toxigenic and non-toxigenic strains were detected (Table 1). Two colonization isolates from one subject in Bulgaria contained *C. difficile* of a previously unknown ribotype which was designated as TechLab RT unknown 36. Of the five subjects who had more than one fecal sample positive for *C. difficile* colonization, four of these had the same ribotype in their second sample, while one subject (503-003) had RT 031 at screening and RT 005 in their 72 h sample suggesting that they may have become colonized with a different RT while in the hospital. Of the seven screening samples positive for *C. difficile*, none were RT 027 or RT 198 and two were non-toxigenic ribotypes. New colonization with RT 027 or 198 was only found in the countries with laboratory-confirmed CDI, and in Bulgaria, where there were no CDI cases, there were also no RT 027 or 0198 among the colonizing isolates.

Despite continuing antimicrobial stewardship efforts and other infection control measures [17], *C. difficile* infection remains a serious health risk, especially for anyone receiving antibiotic therapy. This ribotype analysis supports that ribaxamase may be effective in preventing CDI in areas where epidemic, binary toxin-positive *C. difficile* strains are circulating.

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References

[1] F.C. Lessa, Y. Mu, W.M. Bamberg, Z.G. Beldavs, G.K. Dumyati, J.R. Dunn, M.M. Farley, S.M. Holzbauer, J.I. Meek, E.C. Phipps, L.E. Wilson, L.G. Winston, J.A. Cohen, B.M. Limbago, S.K. Fridkin, D.N. Gerding, L.C. McDonald, Burden of *Clostridium difficile* infection in the United States, N. Engl. J. Med. 372 (2015) 825–834.

[2] V. Stevens, G. Dumyati, L.S. Fine, S.G. Fisher, E. van Wijngaarden, Cumulative antibiotic exposures over time and the risk of *Clostridium difficile* infection, Clin. Infect. Dis. 53 (2011) 42–48.

[3] R.A. Britton, V.B. Young, Role of the intestinal microbiota in resistance to colonization by *Clostridium difficile*, Gastroenterology 146 (2014) 1547–1553.

[4] M. Kaleko, J.A. Bristol, S. Hubert, T. Parsley, G. Widmer, S. Tzipori, P. Subramanian, N. Hasan, P. Koski, J. Kokai-Kun, J. Sliman, A. Jones, S. Connelly, Development of SYN-004, an oral beta-lactamase treatment to protect the gut microbiome from antibiotic-mediated damage and prevent *Clostridium difficile* infection, Anaerobe 41 (2016) 58–67.

[5] J.F. Kokai-Kun, T. Roberts, O. Coughlin, E. Sicard, M. Rufiange, R. Fedorak, C. Carter, M.H. Adams, J. Longstreth, H. Whalen, J. Sliman, The oral beta-lactamase SYN-004 (ribaxamase) degrades ceftriaxone excreted into the intestine in phase 2a clinical studies, Antimicrob. Agents Chemother. 61 (2017).

[6] J.F. Kokai-Kun, T. Roberts, O. Coughlin, H. Whalen, C. Le, C. Da Costa, J. Sliman, ID Week abstract: SYN-004 (ribaxamase) prevents new onset Clostridium difficile infection by protecting the integrity gut microbiome in a phase 2b study, Open Forum Infectious Diseases 4 (2017). S12–S12.

[7] S.L. Stubbs, J.S. Brazier, G.L. O'Neill, B.I. Duerden, PCR targetted to the 16S-23S rRNA gene intergenic spacer region of *Clostridium difficile* and construction of a library consisting of 116 different PCR ribotypes, J. Clin. Microbiol. 37 (1999) 461–463.

[8] B. Geric, R. Carman, M. Rupnik, C. Genheimer, Lyerly D. Sambol, D. Gerding, S. Johnson, Binary toxin–producing, large clostridial toxin–negative *Clostridium difficile* strains are enterotoxic but do not cause disease in hamsters, J. Infect. Dis. 193 (2006) 1143–1150.

[9] M.R. Popoff, E.J. Rubin, D.M. Gill, P. Boquet, Actin-specific ADP-ribosyltransferase produced by a *Clostridium difficile* strain, Infect. Immun. 56 (1988) 2299–2306.

[10] J. Freeman, J. Vernon, S. Pilling, K. Morris, S. Nicholson, S. Shearman, C. Longshaw, M.H. Wilcox, Pan-European Longitudinal Surveillance of Antibiotic Resistance among Prevalent Clostridium difficile Ribotypes Study G, The *Clos*ER study: results from a three-year pan-European longitudinal surveillance of antibiotic resistance among prevalent *Clostridium difficile* ribotypes, 2011-2014, Clin. Microbiol. Infect. (2017), https://doi.org/10.1016/j.cmi.2017.10.008.

[11] E. Valiente, L.F. Dawson, M.D. Cairns, R.A. Stabler, B.W. Wren, Emergence of new PCR ribotypes from the hypervirulent *Clostridium difficile* 027 lineage, J. Med. Microbiol. 61 (2012) 49–56.

[12] N. Shetty, M.W. Wren, P.G. Coen, The role of glutamate dehydrogenase for the detection of *Clostridium difficile* in faecal samples: a meta-analysis, J. Hosp. Infect. 77 (2011) 1–6.

[13] A.C. Labbe, L. Poirier, D. Maccannell, T. Louie, M. Savoie, C. Beliveau, M. Laverdiere, J. Pepin, Clostridium difficile infections in a Canadian tertiary care hospital before and during a regional epidemic associated with the BI/NAP1/027 strain, Antimicrob. Agents Chemother. 52 (2008) 3180–3187.

[14] K. Rao, D. Micic, M. Natarajan, S. Winters, M.J. Kiel, S.T. Walk, K. Santhosh, J.A. Mogle, A.T. Galecki, W. LeBar, P.D. Higgins, V.B. Young, D.M. Aronoff, Clostridium difficile ribotype 027: relationship to age, detectability of toxins A or B in stool with rapid testing, severe infection, and mortality, Clin. Infect. Dis. 61 (2015) 233–241.

[15] M. Krutova, J. Matejkova, P. Drevinek, E.J. Kuijper, O. Nyc, study g, Increasing incidence of *Clostridium difficile* ribotype 001 associated with severe course of the infection and previous fluoroquinolone use in the Czech Republic, 2015, Eur. J. Clin. Microbiol. Infect. Dis. 36 (2017) 2251–2258.

[16] J.T. Wieczorkiewicz, B.K. Lopansri, A. Cheknis, J.R. Osmolski, D.W. Hecht, D.N. Gerding, S. Johnson, Fluoroquinolone and macrolide exposure predict *Clostridium difficile* infection with the highly fluoroquinolone- and macrolide-resistant epidemic *C. difficile* strain BI/NAP1/027, Antimicrob. Agents Chemother. 60 (2016) 418–423.

[17] J.L. Kuntz, D.H. Smith, A.F. Petrik, X. Yang, M.L. Thorp, T. Barton, K. Barton, M. Labreche, S.J. Spindel, E.S. Johnson, Predicting the risk of *Clostridium difficile* Infection upon admission: a score to identify patients for antimicrobial stewardship efforts, Perm. J. 20 (2016) 20–25.