What is the role of free sialic acid in *Clostridium difficile* infection?

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Abstract

In the late 1980s KH Wilson (Duke University) proposed that antibiotics suppress the commensal flora, disrupt normal mucin metabolism, and create excesses of free sialic acid and N-acetylglucosamine (GlcNAc) that *C. difficile* can exploit; without it, *C. difficile* cannot colonize. Our aim was to assess the role of sialic acid in *C. difficile* colonization of susceptible hosts. The mean level of free sialic acid in hamster feces 24 h after clindamycin (10 mg/kg) was nearly twice its pre-clindamycin level ($p=0.06$) but fell ($p<0.05$) to its pre-clindamycin level after dosing with 250 mg of fresh human feces in 1 mL of anaerobic diluent or with *C. difficile* 630 (10^3 spores in 1 mL diluent). Hamsters receiving the fecal transplant were refractory to challenge with *C. difficile* spores; hamsters that did not receive the transplant were susceptible to colonization and diarrhea. As in hamsters, sialic acid levels in feces from healthy humans and those with *C. difficile* diarrhea were similar, suggesting the normal flora, *C. difficile* and an effective probiotic are competitors for the sialic acid. In a molecular study of select components of the normal flora of hamsters before and after clindamycin and *C. difficile*, the most significant finding was the decrease in *Akkermansia muciniphila* levels to below detectable levels within 24 h of the antibiotic. Unlike the smaller and temporary decreases seen with several other groups, including *Clostridium* XIVa which contains many mucin degraders, *A. muciniphila* levels did not recover. An *in vitro* assessment of growth, based on GDH, showed two ribotype 027 isolates of *C. difficile* produced twice the GDH growing on sialic acid than they did on glucose and other sugars. Probing online whole genome sequences of *C. difficile* for sialidase genes shows while other genes of the sialic acid operon are present, the sialidase gene is not, indicating it is the pool of free sialic acid produced by the residual flora, not mucin itself, that is critical to colonization. Once it has bound the free monomer, *C. difficile* grows well on sialic acid. We have confirmed that *C. difficile (Cd)* colonization requires excess free sialic acid. Fecal transplants restore pre-antibiotic levels of sialic acid. This should be a requirement of effective probiotic treatments.

Background

•Healthy gut floras metabolized host-mucin. Mucin was not found in healthy feces. Clindamycin-treated and germ-free floras had reduced mucin metabolism and fecal mucin was present (Carlstadt-Duke, 1989).

•Broth made with fecal filtrates from healthy mice (i.e. no mucin) supported cell division of *C. difficile* in a chemostat but only at a rate below that of wash out. Division occurred, colonization did not. With supplemental sialic acid or GlcNAc growth outpaced wash out and *C. difficile* colonized the chemostat (Wilson & Perini, 1988).

•Other mucin sugar residues did not support colonization nor did supplements of amino acids.

•Wilson proposed that disruption to the orderly release and normal consumption of mucin sugar residues separates unsuccessful from successful colonization bids by *C. difficile.* Antibiotics disturb that balance, creating temporary surpluses of sialic acid an GlcNAc that are exploited by *C. difficile* which, lacking a sialidase of its own, would not normally be available to *C. difficile* (Wilson & Perini, 1988).

•Wilson & Perini (1988) also showed that chemostats inoculated with healthy mouse feces generated protective probiotic floras but only when mucin or sialic acid or GlcNAc was added to the medium. With neither, the chemostat flora was not protective

•Colonization of clindamycin treated hamsters by non-toxigenic isolates was enhanced by pre-treatment with sterile toxic filtrates. (Borriello et al., 1988). The enhancement of colonization by *C. difficile* toxins may have been the result of an increase in available nutrients (Griffin et al., 1986; Linden et al., 2008) complementing the initial increase seen after clindamycin exposure.

- Whole genome sequence analysis shows *Cd* lacks a sialidase but does have enzymes needed for the subsequent uptake, intake and breakdown of sialic acid.

Summary: Colonization with *C. difficile* requires mucin sugars, particularly sialic acid and GlcNAc, to be released by the remnants of the normal gut flora present after antibiotic exposure.

Aims

To assess the "motives", methods and opportunities for the enhanced growth of *Cd* on elevated sialic acid and GlcNAc levels in feces after antibiotic exposure.

- Does clindamycin eliminate mucin metabolizers from the hamster flora?
- Does clindamycin lead to free sialic acid in the ceca of hamsters?
- Is the free-sialic acid pool reduced in hamsters by fecal transplants?
- Do sialic acid or GlcNAc support the growth of *C. difficile*?

Materials and methods

Hamsters: Female hamsters (~100 g) housed individually in micro-isolator cages, were given 10 mg clindamycin/kg orally. 24 h later they were dosed with 10^3 *Cd* spores or sterile buffer. Feces were collected daily. Animals were culled at set intervals and their cecal content was collected *post mortem*. Animals receiving the fecal transplant were given 250 mg fresh human feces in 1 mL of anaerobic diluent 24 h after they were given clindamycin.. The controls received only diluent. All studies were approved by VA Tech IACUC.

Sialic acid assay: Free sialic acid was assayed using the Sialic Acid Quantitation Kit (Sigma-Aldrich).

Human clinical fecal samples: Anonymous, unlinked and already existing clinical samples, each from the same area of southwest Virginia and each containing a toxigenic *Cd*, detectable toxin and GDH were used. TechLab IRB #1 approved the study.

Growth of *Cd* on sialic acid: We inoculated fastidious anaerobe broth (FAB) supplemented with sialic acid or other mucin sugar residues (0.5% wt/vol, the same concentration as glucose in BHI) or mucin (2%) with actively growing *Cd* in FAB. At set intervals we measured GDH, toxin A and toxin B using immunoassays and pure reagents to generate standard curves.

Healthy fecal donor: Feces from a self-reported healthy donor were collected and used within 2 h. TechLab IRB #1 approved the study.

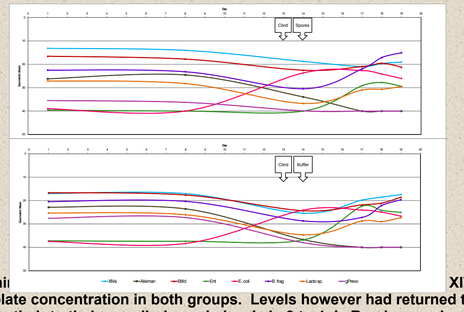
Table 1. qRT PCR sources of methods

TARGET	REFERENCE
Clostridium cluster XIVa	Rinttila et al. 2004 J. Appl. Microbiol 97,1166-1177
Akkermansia muciniphila	Collado, et al. 2007 Appl Environ Microbiol. 7767-7770
Bifidiobacterium spp.	Rinttila et al.2004 J. Appl. Microbiol 97,1166-1177
Enterococcus spp.	Rinttila et al.2004 J. Appl. Microbiol 97,1166-1177
B.fragilis group .	Vanhoutte et al.2006 Appl Environ Microbiol 72:5990-5997 (no G-C clamp)
Lactobacillus spp.	.Haarman, et al.2006 Appl. Enviro. Microbiol.
Escherichia coli	*Selective and Sensitive Method for PCR Amplification of Escherichia coli 16S rRNA Genes in Soil* Sabat, et al. Applied And Environmental Microbiology
Prevotella (group)	*Use of 16S rRNA Gene-Targeted Group-Specific Primers for Real-Time PCR Analysis of Predominant Bacteria in Human Feces.* Matsuki et al. (2004) Appl. Enviro. Microbiol.

- ***Clostridium* cluster XIVa was tested because it includes ruminococci. Ruminococci secrete extracellular sialidases and proteases; they are able to digest mucin fully.**
- ***Bifidobacterium* spp. were assayed because they are present in milk-fed babies that are refractory to diarrhea caused by *C. difficile.***
- ***Lactobacillus spp. were* tested because of their association with milk-fed, healthy babies.**
- **Enterococci were tested because they often occur in samples containing *C. difficile.* Some have been nominated for a probiotic flora**
- ***B. fragilis* group was tested because some bacteroides are said to have probiotic value.**
- ***E. coli* was tested because it was predicted that it can be used as a single species probiotic.**

Figure 1. Effect of clindamycin on select components of the flora

Geometric mean Ct values in qRT PCR analyses of select bacterial groups in hamsters given clindamycin on day 13 and *C. difficile* spores on day 14: Top) Received *C. difficile* spores n=6, Bottom) Only diluent, no spores n=12



•Within … XIVa template concentration in both groups. Levels however had returned to nearly their to their pre-clindamycin levels in 3 to 4 d. Ruminococci are members of *Clostridium* XIVa; they secrete extracellular proteases and mucin glycosidases, including sialidases, that together fully digest mucin. They contribute to the reduced viscosity of the exposed surface of the mucin gel that coats the colonic epithelium.

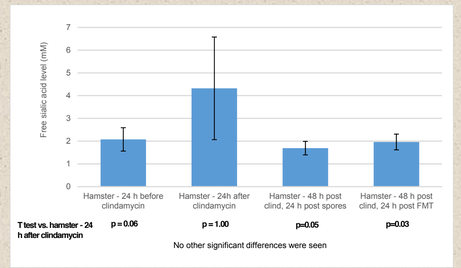
•Clindamycin impacted both *Akkermansia muciniphila* and *Prevotella* spp. Levels. Both declined rapidly to below detectable levels. Their extended absence may have been because the hamsters were housed individually and in microisolators, possibly delaying recolonization from the environment..

•Slightly smaller changes, all temporary, were seen among the bifidobacteria, lactobacilli and bacteroides.

•In both groups, very low levels of endogenous *E. coli* and enterococci rose after clindamycin, the former about 24 h ahead of the latter.

Figure 2. Free sialic acid levels in hamsters: Effects of clindamycin, *C. difficile* and fecal transplant

Cecal contents (n=5/group) were collected within 30 min post mortem and frozen at -70 C until tested.



•Clindamycin exposure caused about a two-fold elevation of free sialic acid levels.

•Inoculation with *C. difficile* or with a fecal suspension (i.e. FMT) reversed this increase.

•FMT protected hamsters from *C. difficile* infection and restored sialic acid to its pre-clindamycin level.

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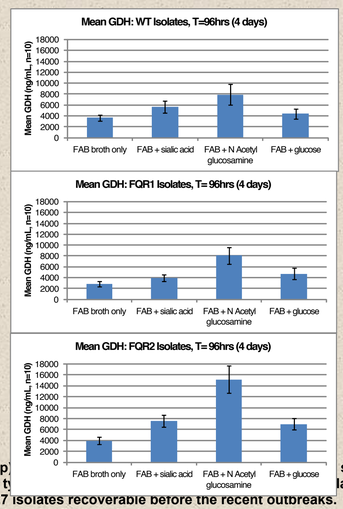
Figure 3. Mucin sugars support the growth of *C. difficile in vitro:* Mean GDH levels generated by growth of C. *difficile* in Fastidious Anaerobe Broth supplemented with mucin sugar residues (n=5 isolates per group and 2 data points per isolate)



•Isolates (n=5 per group) … surrogate marker. One group (WT or wild t… lates of essentially the same lineage as 027 isolates recoverable before the recent outbreaks. They lacked the transposons that, coupled with fluoroquinolone resistance, distinguish the two modern lineages, FQR1 and FQR2 associated with recent outbreaks (He et al., 201). Of the two, FQR2 is the more widespread. Respectively, FQR1 and FQR2 isolates comprised group 2 and 3.

•Wilson (1988) proposed mucin sugar residues as the limiting nutrients in the colonization of mammals by *C. difficile.* In an excess of sialic acid or of GlcNAc *C. difficile,* which lacks a sialidase but has an otherwise functional Nan operon, was able to exploit increased levels of either compound (Figure 3).

•GlcNAc supported significantly more GDH production than sialic acid ($p<0.05$) which itself supported significantly more than did FAB alone. WT and FQR1 isolates produced significantly ($p<0.05$) more GDH in FAB with sialic acid than they did in unsupplemented FAB (Figure 3).

• Utilization of sialic acid and GlcNAc to support rapid growth the rapid growth would be a useful preliminary strategy for *C. difficile* exposed to mucin sugars in the post-antibiotic bowel.

Conclusion

•Wilson's proposal, made over 20 years ago, holds true. Disruption to the orderly release and normal consumption of mucin sugar residues is a tipping point that separates unsuccessful from successful colonization bids by *C. difficile* (Wilson & Perrini, 1988).

•Following antibiotics free mucin sugar residue levels rise and germinating spores exploit this resource. Without antibiotics, residue levels are low and colonization is harder regardless of spore numbers.

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