P0742

Can a human gut model of *Clostridium difficile* infection (CDI) be used to explain GDH-positive/toxin-negative faecal samples seen in clinical practice?

Davies K A[1,2], Chilton C[2], Crowther G[2], Todhunter S[2], Heritage J[3], Wilcox M H[1,2]

[1]Microbiology department, Leeds Teaching Hospitals NHS Trust, Leeds, UK. [2]Leeds Institute for Biomedical and Clinical Sciences, University of Leeds, Leeds, UK. [3]School of Molecular and Cellular Biology, Faculty of Biological Sciences, University of Leeds, Leeds, UK

Introduction

Clostridium difficile is the pathogen most commonly associated with nosocomial intestinal infections[1]. Patients with *C. difficile* infection (CDI) may have mild to severe diarrhoea which can be life threatening[2]. Optimal diagnosis of CDI has recently been reported as glutamate dehydrogenase (GDH) or nucleic acid amplification tests (NAAT) followed by *C. difficile* toxin detection on those samples positive by the first test[3]. This has led to the identification of a new category of patient: GDH-positive/toxin-negative individuals. The clinical relevance of such patients is unclear, as is the relationship between GDH and toxin expression/detection.

A human gut model has been developed which mimics the physiochemical conditions and bacterial populations of the proximal to distal human colon[4]. This model has been used to observe the effects of antibiotics on the growth and toxin production of *C. difficile*; the total viable counts of *C. difficile* and toxin levels rise and consequently fall in-phase with each other during simulated CDI[5].

The aim of this study is to use the human gut model to measure GDH expression during simulated CDI in relation to bacterial growth and toxin production.

Methods

Study design:
A triple-stage weir cascade continuous culture model of *C. difficile* infection (CDI) was monitored for levels of glutamate dehydrogenase (GDH) using a commercial detection kit, to determine if GDH levels varied during CDI.

Continuous culture model of CDI:
A model of the human gut was set up as previously described[5]. Three culture vessels were connected in series, via a weir cascade system, preloaded with human faecal emulsion and drip fed with nitrogen sparged culture media. The weir cascade enables the model to mimic the nutrient and pH changes along the proximal to distal colon of the human gut. Samples were taken from each vessel daily, and cultured on selective media to enumerate faecal bacteria. Population levels were allowed to proliferate and then stabilise (reach steady state) over a roughly two week period. Two models were run in parallel, denoted A and B, which had slightly different treatment schedules.

Once steady state was reached *C. difficile* spores (~10^7 cfu/mL) were added and the model allowed to equilibrate once again. To induce simulated CDI, clindamycin (33.9mg/L QDS) was added to the model, along with another dose of *C. difficile* spores. Development of simulated CDI was monitored by enumeration of total viable counts and spore counts of *C. difficile*, along with the production of *C. difficile* toxin B. Germination was defined as divergent total viable and spore counts of *C. difficile* in conjunction with toxin production. Once germination was seen, a 'treatment' antibiotic was added to the system, and again daily samples were tested for toxin levels, and viable cells and spores of *C. difficile* were enumerated on selective media. Daily samples were frozen at -20 C before further testing.

Glutamate dehydrogenase enzyme immunoassay:
A commercial enzyme immunoassay (EIA) C. DIFF CHEK-60™ (techlab, US) was used to measure the level of GDH in the defrosted daily samples taken from the model. The assay was performed as per manufacturer's instructions. Briefly, samples were diluted 1/4 in kit diluent, mixed using a vortex and spun at 10,000g in a centrifuge to sediment any large particulates. The diluted samples and reagents were loaded onto an automated EIA platform (DS2, Dynex, US) to complete the assay. Results were determined according to the kit cut-off, and both the result and the optical density of each sample were recorded.

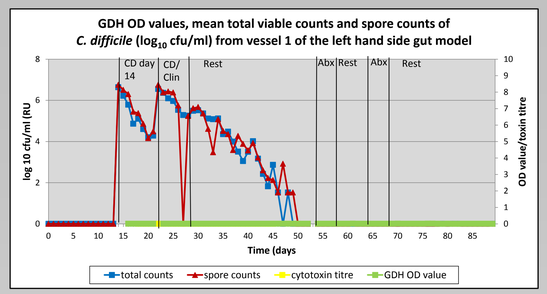
GDH EIA OD values for each daily sample were potted against the *C. difficile* total viable counts, spore counts and toxin production, and compared for patterns of rise and fall.

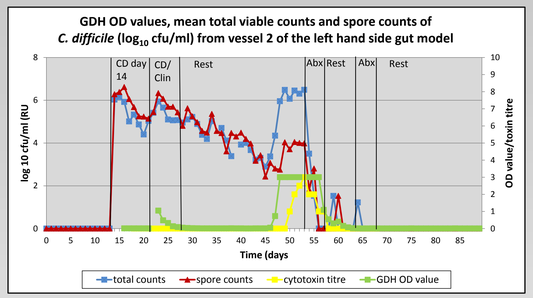
Results

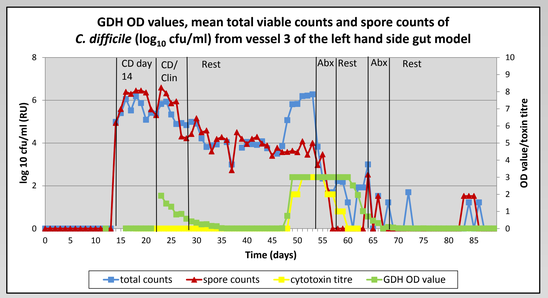
- GDH was detected in vessels 2 and 3 of the model using the commercial assay, but not in vessel 1.
- GDH increased in phase with the total viable counts and toxin titres during germination.
- The levels of GDH reach the 'peak' of the commercial assay during germination.
- GDH levels decreased at the same rate as total viable counts and toxin titres, although there was a lag of 2-3 days.
- GDH was detectable before germination.

Results

Model A

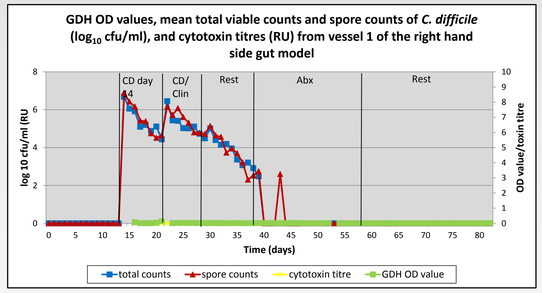


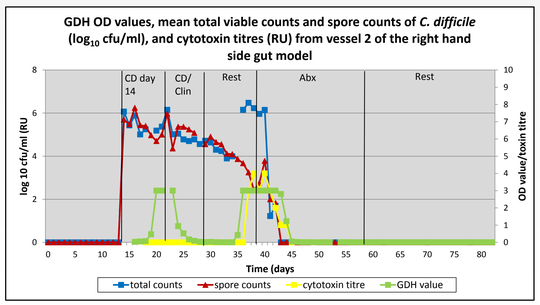


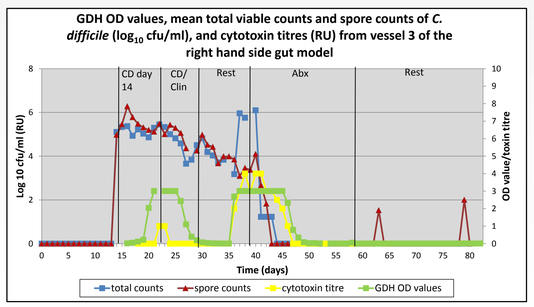


Graphs showing total viable and spore counts of *C. difficile*, toxin (titre) and GDH (EIA OD) production in the three vessels of model A over time. Periods of induction with Clindamycin (Clin) and treatment with another antibiotic (Abx) are shown.

Model B







Graphs showing total viable and spore counts of *C. difficile*, toxin (titre) and GDH (EIA OD) production in the three vessels of model B over time. Periods of induction with Clindamycin (Clin) and treatment with another antibiotic (Abx) are shown.

Discussion

GDH production by *C. difficile* in the human gut model increases in phase with total viable counts and toxin production during simulated CDI. Unfortunately the upper limit of detection of the assay is reached within 2-3 days and the true level of GDH cannot be determined. Further investigations are needed to determine whether the assay is quantitative, which would allow a dilution step to be used to determine the 'true' level of GDH produced.

Once reached, the peak level of GDH remains in the system for longer than the peak level of both total viable counts and toxin. The reasons for this may be multifactorial; GDH may be a very stable protein that is not easily broken down in the system, it may be sequestered in biofilm within the model or dying cells within the model may release large amounts of the protein which effectively 'flood' the system with GDH. Elucidating the 'true' peak levels of GDH produced may resolve some of these issues. Additionally, sequestration of GDH into biofilm will be investigated using a biofilm human gut model[6].

GDH was not detected in vessel one in either model. This would appear to correlate with the lack of *C. difficile* proliferation in vessel one, as shown by total viable counts. It is possible however, that the assay might be affected by the acidic pH in this vessel, which may have compromised the 3-dimensional composition of the protein.

Increases in GDH were also detected outside of simulated CDI. After introduction of spores to the system, increases in GDH, up to the peak of the assay, were measured in both models. This could be the result of the organism reaching equilibrium within the model; although this does not appear to coincide with an increase in total viable counts as might be expected. Although this increase in GDH is only seen once in the model, it is possible that it may occur several times if the time between introduction of the spores and induction of CDI was extended.

There are therefore two points within the model that GDH is detected without the presence of increased total viable counts or toxin production; before induction of CDI and after treatment of CDI. Does this give a possible explanation for some of the GDH positive/toxin negative results found in some patients? While some of these results can be explained by carriage of non-toxigenic strains or sub-optimal toxin assays[3], it is possible that proliferation of *C. difficile* is occurring without onset of CDI, and that this is being detected by the GDH assay. Perhaps, therefore, GDH can be used to detect patients in the early stages of colonisation with CDI or recovering from disease.

Conclusions

- GDH levels in the gut model can rise without a concurrent increase in toxin levels or cell counts.
- Additionally, a high level of GDH can remain in the system while toxin levels and viable cell counts decrease. GDH-positive/toxin-negative clinical sample results could be explained by this model.
- Potentially, patients who have primary *C. difficile* colonisation, or who have cleared/are resolving CDI may have raised levels of GDH in the absence of detectable toxin.
- Prospective studies in patients are needed to validate whether these data for *C. difficile* diagnostics are seen *in vivo*.

References

1. European Centre for Disease Prevention and Control point prevalence survey of healthcare-associated infections and antimicrobial use in European acute care hospitals 2011-2012 http://www.ecdc.europa.eu/en/publications/_layouts/forms/Publication_DispForm.aspx?List=4f55ad51-4aed-4d32-b960-af70113dbb90&ID=865. Accessed 11th April 2014
2. Crobach M J, Dekkers O M, Wilcox M H and Kuijper E J. European society of clinical microbiology and infectious diseases (ESCMID): Data review and recommendations for diagnosing *Clostridium difficile*-infection (CDI). 2009. *Clinical Microbiology and Infection*. 15 (12):1053-1066
3. Planche T D, Davies K A, Coen P G, Finney J M, Monahan I M, Morris K A, O'Connor L, Oakley S J, Pope C F, Wren M W, Shetty N P, Crook D W, Wilcox M H. Differences in outcome according to *Clostridium difficile* testing method: a prospective multicentre diagnostic validation study of *C difficile* infection. 2013. *Lancet Infectious Diseases*.13(11):936-45.
4. Freeman J, Baines S D, Saxton K, Wilcox M H. Effect of metronidazole on growth and toxin production by epidemic *Clostridium difficile* PCR ribotypes 001 and 027 in a human gut model. 2007. *Journal of Antimicrobial Chemotherapy*. 60(1):83-91.
5. Freeman J, O'Neill F J, Wilcox M H. Effects of cefotaxime and desacetylcefotaxime upon *Clostridium difficile* proliferation and toxin production in a triple-stage chemostat model of the human gut.2003. *Journal of Antimicrobial Chemotherapy*. 52(1):96-102. Epub 2003 May 29
6. Crowther GS, Chilton CH, Todhunter SL, Nicholson S, Freeman J, Baines SD, Wilcox MH. Development and validation of a chemostat gut model to study both planktonic and biofilm modes of growth of *Clostridium difficile* and human microbiota. 2014. *PLoS One*. 2014 Feb 6;9(2):e88396. doi: 10.1371/journal.pone.0088396. eCollection 2014. Accessed 22nd April 2014

Acknowledgements

This work forms part of a PhD for Kerrie Davies and is funded by Public Health England