**Supplemental Digital Content 2.** Molecular methods for *C. difficile* testing

A multiplex PCR assay targeting a species-specific internal fragment of the triose phosphate isomerase (tpi) housekeeping gene, internal core sequences of both toxins A (tcdA) and B (tcdB) genes was used to confirm all isolates as C. difficile and determine toxigenicity.1

*C. difficile* isolates were typed by PCR-ribotyping according to the method described by Bidet et al2 and compared to a panel of the UK’s commonest circulating ribotypes.3 Any ribotypes which could not be identified were sent to the Section of Experimental Bacteriology, Leiden University, Netherlands for confirmatory testing.

DNA was extracted from 59 stool samples with sufficient sample remaining, using the Stratec PSP Spin Stool DNA Plus kit (Stratec biomedical, Berlin, Germany), according to the manufacturer’s instructions.

All of those who were both *C. difficile* antigen and toxin positive, *C. difficile* antigen positive and toxin negative, and an age-matched group who were *C. difficile* antigen and toxin negative underwent DNA isolation and amplification of the V4 region as follows. Dual index amplicon library was constructed with a 2 step PCR protocol, the first selecting the V4 region for 16S sequencing,4,5 with PCR amplification being performed on a Mastercycler apparatus (Eppendorf, Hamburg, Germany). Clean-up was performed using AMPure XP magnetic beads (0.8:1) (Roche, Basel, Switzerland) according to the supplier's instructions. Second stage PCR was required for index read preparation, and a second clean-up using AMPure beads was performed. Amplicon library assessment was performed on the 2100 Bioanalyzer using the High Sensitivity DNA kit (Agilent Technologies, Waldbronn, Germany). After normalizing and pooling the libraries the pools were then size selected with Pippen Prep (Sage Science, USA) for the V4 region (432bp). Sufficient reads for analysis were obtained from 42 samples (18 *C. difficile* culture negative, 9 culture positive/toxin negative,15 culture positive/toxin positive) for comparison of microbiome composition and diversity index. Sequencing was performed at the Centre for Genomic Research, Liverpool. Quality and quantity of the final library was assessed using the Illumina Library Quantification Kit from Kapa on a Roche Light Cycler LC480II, according to the manufacturer’s instructions.

*Alternative pathogen testing*

In order to investigate for the presence of co-infections and determine likely cause of diarrhea in these patients, extracted DNA from the same 59 stool samples was subject to a nucleic acid amplification-based test (NAAT) using the Luminex xTAG© gastrointestinal pathogen panel (Luminex, Austin, TX, USA) as per the manufacturer’s instructions. This panel is able to detect 15 gastrointestinal pathogens, including bacteria (*Campylobacter*, *C. difficile*, *E. coli* 0157, Enterotoxigenic *E. coli*, Shiga-like toxin producing *E. coli*, *Salmonella*, *Shigella*, *Vibrio cholera*, *Yersinia enterocolitica)*, viruses (*Adenovirus, Norovirus, Rotavirus A)* and parasites (*Cryptosporidium, Entamoeba histolytica, Giardia).*

**Statistics**

*Analysis of 16S sequencing data*

Initial processing and quality assessment of the sequencing data was done by base calling and de-multiplexing of indexed reads using CASAVA version 1.8.2 (Illumina) to produce 42 samples from the 2 lanes of sequence data in fastq format. The raw fastq files were re-trimmed to remove Illumina adapter sequences using Cutadapt version 1.2.1 (Martin, 2011). The option “-O 3” was set, so the 3’ end of any reads which matched the adapter sequence over at least 3bp was trimmed off. The reads were further trimmed to remove low quality bases, using Sickle version 1.200 with a minimum window quality score of 20. After trimming, reads shorter than 10bp were removed. If both reads from a pair passed this filter, each was included in the R1 (forward reads) or R2 (reverse reads) file. If only one of a read pair passed this filter, it was included in the R0 (unpaired reads) file.

Read pairs were aligned in order to produce a single sequence for each read pair that would entirely span the amplicon. FLASh version 1.2.8 (Magoc and Salzberg, 2011) was used to assemble each pair of reads into a single sequence representing the complete amplicon. Fragmented PhiX phage was added to the sequence library in order to increase the sequence complexity. Each sample was compared with the complete PhiX sequence (GenBank gi9626372) using BLASTn (Altschul et al. 1990). Sequences matching PhiX (E-value <10-5) were filtered out of the dataset. The final preparatory step was the exclusion of sequences with lengths outside an expected range (which are likely to represent errors), a custom script was used to remove sequences shorter than 200bp or longer than 300bp (i.e. expected amplicon length +/- 50bp). The sequences for each sample were pooled into a single file and a metadata file created to describe each sample. These two files were used for metagenomic analysis using Qiime, version 1.8.0 (Caporaso et al. 2010) and the Greengenes 13.8 database of ribosomal RNA genes.

**References**

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