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Evaluation of bacteriophage therapy to control *Clostridium difficile* and toxin production in an *in vitro* human colon model system

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ABSTRACT

Clostridium difficile is a leading cause of hospital-acquired diarrhoea and represents a major challenge for healthcare providers. Due to the decreasing efficacy and associated problems of antibiotic therapy there is a need for synergistic and alternative treatments. In this study we investigated the use of a specific bacteriophage, ΦCD27, in a human colon model of *C. difficile* infection. Our findings demonstrate a significant reduction in the burden of *C. difficile* cells and toxin production with phage treatment relative to an untreated control, with no detrimental effect on commensal bacterial populations. The results demonstrate the potential of phage therapy, and highlight the limitations of using phages that have lysogenic capacity.

1. Introduction

Clostridium difficile remains a leading cause of nosocomial infections in England and Wales, despite an encouraging decline in the number of reports since 2007 [1]. The onset of *C. difficile* infection (CDI) is highly associated with the use of broad-spectrum antibiotics and the resulting disruption to populations of resident microbes (the microbiota) [2].

C. difficile infection requires treatment, for which metronidazole is commonly used. Resistance to this agent has been reported [3] and the incidence of treatment failures has increased in recent years [4]. The use of metronidazole has also been implicated as a cause of CDI [5]. Vancomycin is an alternative antibiotic, although its use is tightly restricted to help mitigate against the development of resistance [6]. There is therefore a need for alternative drugs and treatments. Bacteriophage (phage) therapy offers one possible approach.

The use of bacteriophages as therapeutic agents began shortly after their discovery in the early 20th century [7]. In Western countries phage therapy was superseded by the widespread availability of penicillin but there is currently renewed interest in view of the need to overcome the problem of multi-resistant bacterial pathogens [8]. A number of phages infecting *C. difficile* have been described to date, all of which contain typical elements associated with lysogeny control, including repressors, anti-repressors and integrases [9–14]. The apparent absence of phages with a solely lytic lifestyle could be due to the high incidence of prophages in *C. difficile* genomes, which may impart resistance to further infection [9,15]. Also, a lysogenic relationship is likely to be favoured in hosts that have the capacity for sporulation, as is the case with *C. difficile*.

Previously we isolated and characterised ΦCD27, a bacteriophage belonging to the Myoviridae family [13]. In batch fermentation models of CDI, prophylactic treatment with ΦCD27 resulted in a significant reduction of viable *C. difficile* cells with no detrimental impact on the main bacterial groups of the colonic microbiota [16]. Our current study builds on this work, by simulating the microbiological aspects of *C. difficile* infection from colonisation to fulminant disease using a human colon model that correlates well with the findings from *in vivo* studies [17,18] to measure the effectiveness of prophylactic phage therapy using ΦCD27 over a period of 35 days.

2. Materials and methods

2.1. Bacteria and bacteriophage strains

C. difficile strains NCTC 11204 and 12726 were obtained from the National Collection of Type Cultures (PHL, London), maintained in

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Robertson's cooked meat media (SGL, Corby) at room temperature and grown anaerobically at 37 °C in pre-reduced brain heart infusion (BHI) broth (Oxoid, UK) supplemented with vitamin K_1 (50 μg/L), haemin (5 mg/L), resazurin (1 mg/L), and L-cysteine (0.5 g/L). A spore preparation was made by growing cultures on blood agar (Oxoid) under anaerobic conditions for 10 d at 37 °C and then exposing to air for 2 d. Subsequently cells were harvested, washed and re-suspended in 1 mL PBS. An equal volume of 96% ethanol was added before incubation on ice for 1 h. Finally, the samples were washed 4 times, resuspended in PBS and enumerated using cyclosporine cefoxitin egg-yolk (CCEY) agar (Bioconnections Ltd.) and the volume adjusted to give a concentration of approximately 1×10^7 CFU/mL. *Micrococcus luteus* FI10640 (in-house strain collection) was used for the clindamycin bioassays and was grown anaerobically at 37 °C in MRS broth (Oxoid).

Bacteriophage ΦCD27 was induced from *C. difficile* NCTC 12727 using mitomycin C (Sigma), as described previously [16]. Phage stocks were propagated by recovering phage particles from semi-confluent plaque assays. 15 mL of BHI broth was added to each plate, covered with foil and rotated gently at ambient temperature for 3–4 h. The medium was recovered and filtered through a 0.45 μm filter (Sartorius Ltd.) and the preparation was enumerated by overlay plaque assay [16]. Volumes were adjusted to give a concentration of approximately 1×10^8 PFU/mL. Fresh phage stocks were produced for each experiment. For the phage-free control broth, 15 mL BHI was added to non-infected lawns of *C. difficile*, incubated and harvested as described above.

2.2. The three-component continuous colon model system

The method of Freeman et al. [17] was used to produce an *in vitro* model of *C. difficile* infection, using clindamycin treatment in a colon model previously validated by Macfarlane et al. [18]. Freshly voided faeces from the same healthy male volunteer aged over 60 years were used to prime the system in each replicate experiment. Faecal suspensions were made with an equal volume of pre-reduced PBS, homogenised using a stomacher (Seward Ltd.) at 230 rpm for 45 s and comprised 50% of the volume of each vessel. The faecal inoculates were tested to confirm the absence of indigenous *C. difficile*.

Each experiment comprised 2 model systems – one to serve as a control and one to receive the prophylactic phage therapy. In the prophylactic system, phage preparations were dosed at a multiplicity of infection of 10 and were dispensed into vessel 1 (ascending section) every day from day 1 until the end of the experiment. The same volume of phage-free broth was administered to the control system. Samples (5 mL) were taken from all vessels of each system approximately every 3 d. Colon models were treated with 33.9 mg/L clindamycin (Melford Laboratories) every 6–8 h between day 14 and day 21. This disrupted the normal levels of faecal bacteria permitting the germination and growth of *C. difficile,* and mimicking *in vivo* disease. To confirm that the correct concentration of clindamycin was maintained in the system, bioassays were carried out on filtered supernatants of colon model samples on days 1, 3 and 7 of the antibiotic course, using *M. luteus* as the indicator.

2.3. Bacterial enumerations

All bacterial counts were performed in triplicate using 20 μl samples of both undiluted and 10-fold dilutions in pre-reduced PBS. Vegetative cells of *C. difficile* were enumerated on pre-reduced CCEY agar under anaerobic conditions. Spores of *C. difficile* were enumerated by exposure of a sample to an equal volume of 96% ethanol for 1 h at ambient temperature, followed by four washes in PBS. Suspensions were serially diluted in PBS and plated onto CCEY agar. The levels of other gut bacteria were evaluated by culturing on selective media as described previously [16]. The area under the growth curve was calculated to represent the overall levels of growth [16]. All replicates of the control and the phage-treated system were tested by means of a one-tailed paired *t* test.

2.4. Influence of C. difficile toxins

Enumerations of specific groups of commensal bacteria were carried out from cultures of faecal sample inoculated with *C. difficile* NCTC 11204 (a toxigenic strain) and NCTC 12726 (a non-toxigenic strain) to ascertain possible effects of *C. difficile* toxins on the growth of common groups of commensal bacteria. Similarly, faecal cultures in the presence and absence of *C. difficile* cells which had been autoclaved then disrupted (by bead-beating using the Fast-Prep FP 120 cell disruptor for 4 cycles of 30 s), were used to investigate possible effects of the cell lysate on commensal bacteria.

2.5. Denaturing gradient gel electrophoresis (DGGE)

Molecular profiles of the gut bacteria from the control and phage-treated systems were also obtained using DGGE as described previously with primers targeting the V3 region of 16S rDNA [19]. PCR reactions used colon model samples directly after a freeze–thaw cycle and were supplemented with 0.2 μg/μL BSA. Analysis was performed as detailed previously [19].

2.6. Toxin quantification

Premier Toxins A & B immunoassay kits (Meridian Bioscience) were used according to the manufacturer's instructions to quantify the combined production of *C. difficile* toxins A and B in each vessel of the colon model. Results were read using the Thermo Max Microplate Reader with a single wavelength of 450 nm.

2.7. Detection of prophage

Isolates of *C. difficile* from the colon model systems at day 35 of the experiments were grown for 12–18 h in pre-reduced BHI broth with supplements. Mitomycin C-induced cultures were filtered (0.45 μm), spotted onto BHI agar plates seeded with *C. difficile* (NCTC 11204), and incubated overnight to assess plaque development.

3. Results

3.1. The effect of phage therapy on vegetative cells and spores of C. difficile

A continuous colon model system was used to investigate the effect of prophylactic phage therapy on *C. difficile* growth and toxin production. A significant decrease in the total number of *C. difficile* vegetative cells was recorded in the phage-treated systems relative to the untreated controls ($p = < 0.001$). In samples from the phage-treated vessels of the first replicate, *C. difficile* was not detected, whereas in the control vessels high numbers ($>10^5$ CFU/mL) were recorded less than 24 h after the clindamycin treatment ceased (Fig. 1A). The absence of vegetative *C. difficile* cells in samples from the phage-treated systems was confounded by the significant increase in the number of spores compared to the control ($p = < 0.001$) (Fig. 2A). In replicate 2, phage treatment was not successful at preventing the proliferation of *C. difficile* and levels comparable to that of the control system were recorded between days 24 and 28 (Fig. 1B). Interestingly, on this occasion the phage treatment yielded significantly fewer spores relative to the control