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Research Paper

Oral Phage Therapy of Acute Bacterial Diarrhea With Two Coliphage Preparations: A Randomized Trial in Children From Bangladesh

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ABSTRACT

Background: Antibiotic resistance is rising in important bacterial pathogens. Phage therapy (PT), the use of bacterial viruses infecting the pathogen in a species-specific way, is a potential alternative.
Method: T4-like coliphages or a commercial Russian coliphage product or placebo was orally given over 4 days to Bangladeshi children hospitalized with acute bacterial diarrhea. Safety of oral phage was assessed clinically and by functional tests; coliphage and *Escherichia coli* titers and enteropathogens were determined in stool and quantitative diarrhea parameters (stool output, stool frequency) were measured. Stool microbiota was studied by 16S rRNA gene sequencing; the genomes of four fecal *Streptococcus* isolates were sequenced.
Findings: No adverse events attributable to oral phage application were observed (primary safety outcome). Fecal coliphage was increased in treated over control children, but the titers did not show substantial intestinal phage replication (secondary microbiology outcome). 60% of the children suffered from a microbiologically proven *E. coli* diarrhea; the most frequent diagnosis was ETEC infections. Bacterial co-pathogens were also detected. Half of the patients contained phage-susceptible *E. coli* colonies in the stool. *E. coli* represented less than 5% of fecal bacteria. Stool ETEC titers showed only a short-lived peak and were otherwise close to the replication threshold determined for T4 phage *in vitro*. An interim analysis after the enrollment of 120 patients showed no amelioration in quantitative diarrhea parameter by PT over standard care (tertiary clinical outcome). Stool microbiota was characterized by an overgrowth with *Streptococcus* belonging to the *Streptococcus gallolyticus* and *Streptococcus salivarius* species groups, their abundance correlated with quantitative diarrhea outcome, but genome sequencing did not identify virulence genes.
Interpretation: Oral coliphages showed a safe gut transit in children, but failed to achieve intestinal amplification and to improve diarrhea outcome, possibly due to insufficient phage coverage and too low *E. coli* pathogen titers requiring higher oral phage doses. More knowledge is needed on *in vivo* phage–bacterium interaction and the role of *E. coli* in childhood diarrhea for successful PT.
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1. Introduction

In view of the growing threat of antibiotic resistance, WHO has warned for a return into a pre-antibiotic era (Martinez, 2012). Alternatives to antibiotics are thus urgently needed (Piddock, 2012; Stanton, 2013). Phage therapy (PT), the use of bacterial viruses (phages) against pathogens, is a potentially attractive option for the prevention and treatment of some bacterial infections (Sulakvelidze et al., 2001; Brüssow, 2005, 2012). Indeed, a large, randomized and placebo-controlled trial conducted in children from Tbilisi/Republic of Georgia during the 1960s

Abbreviations: Cfu, colony forming unit; ETEC, enterotoxigenic *E. coli*; EPEC, enteropathogenic *E. coli*; EAEC, enteroaggregative *E. coli*; M, ColiProteus phage cocktail from Microgen; ORS, oral rehydration solution; P, placebo; pfu, plaque forming unit; PT, phage therapy; qPCR, quantitative polymerase chain reaction; RCT, randomized controlled trial; T, T4 phage cocktail from NRC.

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described prevention of *Shigella* dysentery and *Escherichia coli* diarrhea with orally applied *Shigella* phages (Sulakvelidze et al., 2001). PT was practiced in Poland in patients with antibiotic-resistant bacterial infections on a case-by-case basis, apparently with high success (Międzybrodzki et al., 2012). However, randomized controlled trials (RCTs) in humans are needed to rationally assess the potential of PT. So far, a single small RCT documented an effect in carefully selected patients suffering from otitis externa ("swimmer's ear") (Wright et al., 2009).

E. coli is an important cause of diarrhea in children from developing countries (Qadri et al., 2005), resistant against many antibiotics (Jiang et al., 2002) while efficient *E. coli* diarrhea vaccines are not yet available (Ahmed et al., 2013). Other than zinc and oral rehydration solution, no specific, effective, safe and affordable treatment is available to reduce the severity or duration of illness caused by this bacterial agent. We hypothesize that oral administration of coliphage will be effective in reducing severity of diarrhea in children with proven *E. coli* induced diarrhea. Therefore a RCT of PT with two different coliphage cocktails for which safe use was demonstrated in healthy adults (Sarker et al., 2012; McCallin et al., 2013) was performed in children with *E. coli* diarrhea. The primary endpoint was the safety of oral coliphage in children infected with *E. coli*, the secondary endpoint was the titration of fecal coliphage and *E. coli* pathogen to assess *in vivo* lytic phage activity and the tertiary endpoint was the impact of oral phage on quantitative diarrhea parameters. Oral coliphage reached the intestine, but did not achieve treatment effects over placebo most likely because intestinal *E. coli* titers were low and close to the replication threshold of coliphages. Microbiota analysis revealed a marked outgrowth of fecal streptococci during the acute phase of diarrhea.

2. Methods

2.1. Participants and Study Design

A prospective, single center, randomized, placebo-controlled, parallel group clinical trial was undertaken to assess the safety and efficacy of T4-like phage cocktail compared to Microgen ColiProteus phage cocktail or placebo in 6–24-month-old male (to obtain stool without urine contamination) children presenting with acute onset of dehydrating diarrhea of less than 48 h duration. The study took place at the Dhaka Hospital of the International Centre for Diarrheal Disease Research, Bangladesh (icddr,b) between June 2009 and September 2011. The trial was approved by the Research Review Committee and Ethical Review Committee of icddr,b (protocol #2008-062) and registered with the Identifier NCT00937274 at the ClinicalTrials.gov site. The study was performed according to Good Clinical Practice and the Declaration of Helsinki (http://www.who.int/bulletin/archives/79(4)373.pdf). Written informed consent was obtained from parents or legal guardians of the child before study enrollment. The T4 phage cocktail contained eleven T4-like phages (AB2, 4, 6, 11, 46, 50, 55; JS34, 37, 98, D1.4) (Bourdin et al., 2014a,b). The composition of the Microgen ColiProteus phage cocktail was described previously by metagenome sequencing and electron microscopy (McCallin et al., 2013). The placebo was reduced osmolarity oral rehydration solution (ORS) supplemented with zinc, the standard treatment for uncomplicated watery diarrhea at icddr,b. The primary outcome of the safety part of the study was the acceptability of the phage products by the patients as determined by clinical observation and liver, renal and hematology tests described previously (Sarker et al., 2012; McCallin et al., 2013). The secondary endpoint was the determination of coliphage and total *E. coli* titers and their phage susceptibility in stool and the clearance of ETEC from stool. The tertiary outcome was changes in total stool output (expressed as g/kg of body weight per day), frequency of stool (number/day) and ORS need for rehydration (in ml/kg body weight per day) from randomization to resolution of illness.

Children with at least four liquid stools during the previous 24 h, with some degree of dehydration (WHO methodology) were considered for study enrollment after obtaining informed consent.

2.2. Randomization

A random permuted block design (with block size of 6) (to equalize the number of patients in the two treatment groups after short intervals) was used for allocation of patients to the study interventions, namely the two active products, *i.e.* T4 coliphage cocktail, Microgen phage cocktail in ORS or placebo (0.9% NaCl) at a 1:1:1 ratio. The icddr,b hospital pharmacist, not associated with the study in any other way, prepared the products and handed it over to the study nurse unaware of the product for dispensing to the patients following a computer-generated list of random numbers developed by an independent biostatistician not associated with the study. Code envelopes were kept by the sponsor and by the investigator for un-blinding in emergency situations and after the blind review meeting. The subjects, principal investigator, hospital staff and laboratory personnel were masked to the treatment assignments. After mixing phage or placebo with ORS powders in 30 ml mineral water (Vittel mineral water, pH 7.6, bicarbonate 384 mg/l) and a food dye, the interventions products were indistinguishable. The study products were given 3 times per day (8 am, noon, 4 pm) for four days.

2.3. Power Calculation

We estimated the sample size based on stool output. In a previous trial at icddr,b in children hospitalized with untreated ETEC and EPEC diarrhea we observed a stool output of 176 + 100 ml/kg body weight in a 4-day period (Casswall et al., 2000). With an anticipated 30% reduction in stool volume, we estimated a sample size of 71 at 5% significance level and 80% power. To adjust for a 5% drop-out rate, we decided to enroll 225 children into the trial. Note that the interim analysis was done with 120 patients, 53% of the planned enrollment.

2.4. Procedures

Following confirmation of the absence of rotavirus in stool by ELISA and *Vibrio* by dark field microcopy, and sending stool samples for further microbiological assessments, *i.e. Salmonella*, *Shigella*, *Aeromonas* and *Campylobacter* according to standard procedures at icddr,b (Harris et al., 2008) and for identification of ETEC, EPEC or EAEC by multiplex PCR assay using specific primers as described (Svenungsson et al., 2000), the enrolled children were randomized to intervention. Children with systemic infection, invasive diarrhea, severe acute malnutrition (weight for height z score < -3SD of WHO median or presence of edema), significant medical abnormalities and who had received or needed antibiotic treatment were excluded. Initial correction of dehydration was performed using hypo-osmolar ORS solution or initial intravenous rehydration solution (for children with severe dehydration) followed by ORS solution, equal to the amount of abnormal (watery or liquid) stool loss, until diarrhea resolves.

Children whose fecal culture identified other bacterial enteric pathogens or no *E. coli* pathogen remained in the study and their data were used for Intention-to-Treat (ITT) analysis. Sub-group analysis was done for confirmed *E. coli* infection. Freshly passed stool was obtained daily at about 4 pm (the time was kept fixed as the first dose was fed exactly at 4 pm) for quantitative colony counts of *E. coli* on EMB and MacConkey agar plates, total bacterial count by qPCR (Nadkarni et al., 2002) and microbiota analysis. Three putative *E. coli* colonies from day 1 stool agar plates were tested for sensitivity to the applied phage cocktails at NRC, Switzerland.

The nursing staff recorded the frequency, consistency and volume of stool and urine passed every six hours (to a sensitivity of 1 g). This was achieved through a combination of placing the subject on a cholera cot and collecting urine in a pediatric urine collection bag. The data collected from this procedure was also used to calculate amounts of ORS solution required to account for fluid losses due to diarrhea. Stool frequency was counted by the study nurse and consistency of every stool passed