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Safety analysis of a Russian phage cocktail: From MetaGenomic analysis to oral application in healthy human subjects

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ABSTRACT

Phage therapy has a long tradition in Eastern Europe, where preparations are comprised of complex phage cocktails whose compositions have not been described. We investigated the composition of a phage cocktail from the Russian pharmaceutical company Microgen targeting *Escherichia coli*/*Proteus* infections. Electron microscopy identified six phage types, with numerically T7-like phages dominating over T4-like phages. A metagenomic approach using taxonomical classification, reference mapping and *de novo* assembly identified 18 distinct phage types, including 7 genera of Podoviridae, 2 established and 2 proposed genera of Myoviridae, and 2 genera of Siphoviridae. *De novo* assembly yielded 7 contigs greater than 30 kb, including a 147-kb Myovirus genome and a 42-kb genome of a potentially new phage. Bioinformatic analysis did not reveal undesired genes and a small human volunteer trial did not associate adverse effects with oral phage exposure.

Introduction

The World Health Organization has officially announced an antibiotic crisis. The crisis has two aspects: on one side there are an ever increasing number of important human bacterial pathogens becoming resistant to antibiotics, and on the other side there are less and less new antibiotics developed by the pharmaceutical industry. Alternatives to antibiotics are thus urgently needed (WHO, 2012). A potentially promising treatment and prevention mode is bacteriophage therapy (Brüssow et al., 2012; Merabishvili et al., 2009; Rhoads et al., 2009; Smith and Huggins, 1982; Sulakvelidze et al., 2001; Wright et al., 2009). Bacteriophages are viruses infecting bacteria and, due to their lytic activity on their host cells, phages have already been explored as therapeutic agents by Félix d'Herelle, who discovered phage nearly a century ago at the Pasteur Institute in Paris. With a Georgian collaborator, Eliava, he went to the Soviet Union, where he founded a large applied phage institute in Tbilisi. The Eliava Institute became the cradle of Soviet phage therapy and, during its heydays, provided therapeutic phages at an industrial scale to the Red Army (Häusler, 2007). With the disintegration of the Soviet Union, the tradition of the Eliava Institute was taken up by Russian pharmaceutical companies, which currently provide the public sector with over-the-counter phage products sold in pharmacies as a registered product. The Russian company Microgen sells phages as liquid preparations or as pressed pills for a number of infections (http://eng.microgen.ru/catalog/). However, no detailed scientific reports describe their safe use in healthy subjects, let alone their safety and efficacy in patients (Sulakvelidze et al., 2001; Sulakvelidze and Kutter, 2005; Brüssow, 2005). Not even the composition of these Russian phage preparations has been described in scientific reports. Here we investigated the Microgen ColiProteus phage preparation against *E. coli*/*Proteus* infection independently from any information about the cocktail composition from the supplier.

Metagenomic analysis is becoming increasingly frequently employed for the investigation of viral populations in both environmental and clinical samples (Breitbart et al. 2002; Ng et al., 2011; Pride et al., 2012; Reyes et al., 2010, 2012; Tse et al., 2012;

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Willner et al., 2009; Victoria et al., 2009). A common strategy to characterize such data sets involves *de novo* assembly of contigs directly from sequencing reads, followed by extensive homology searches. However, closely related species within a sample can create difficulties when using this methodology, and the choice of appropriate assembler programs and alignment parameters is important (Teeling and Glöckner, 2012; Breitbart et al., 2002). Here we analyzed sequencing data first by taxonomical classification of reads using the MetaGenomic ANalyser (MEGAN) (Huson and Mitra, 2012) and reference mapping. These two assembly-independent methods were followed by a *de novo* assembly of unmapped reads to identify potentially new phage genomes. We then used bioinformatic methods to establish the genetic safety of this phage cocktail (Brüssow et al., 2004; Brüssow 2010) and demonstrated their biological safety in a small human volunteer trial.

Results

Electron microscopy

We concentrated phages by a combination of medium and high-speed centrifugation steps directly from the commercial phage preparation. Next to small membrane debris from lysed bacteria, these preparations showed two main constituents: Myoviruses with prolate heads that morphologically resembled T4-like phages and Podoviruses resembling T7-like phages, which were numerically the most prominent phage in the cocktail (Fig. 1A). With lower numbers, we also observed tailed phages with isometric capsids of different diameters and distinct tail and baseplates structures (Fig. 1B–G).

MetaGenomic sequencing and taxonomic classification

The workflow for the analysis of the DNA sequences from the Microgen ColiProteus cocktail is described in Fig. 2. In a first step, a crude virus pellet was isolated from the Microgen cocktail by differential centrifugation and the total viral DNA was extracted and sequenced with the Illumina HiSeq 2000 technology, yielding 16.4 million paired-end reads of 100 bp. After filtering for low quality and low complexity sequences, 3.2% of reads were removed. When removing redundancy at 100% identity, the read set decreased to 3.6 million non-redundant (nr) paired-end reads. This nr set was used for safety evaluation and reference mapping.

To permit time-consuming blastx analysis, we then removed redundancy at 95% identity to locate divergent reads. This further reduced the set to 0.6 million reads. These reads were compared against the NCBI-NR protein database.

All blastx files were then imported into MEGAN, which assigns hits to taxa using the Lowest Common Ancestor (LCA) algorithm, in order to visualize the taxonomic content of the reads (Huson and Mitra, 2012). Reads were classified in MEGAN as Myoviridae (34%), Podoviridae (24%), Siphoviridae (6%), unclassified phage (1%), and as "bacterial" DNA (10%), while 23% showed no hits (Fig. 3A).

Reference mapping

Reference genomes closely related to phages contained in the cocktail were identified by increasing the stringency parameters in MEGAN (Supplementary Fig. 1). Of the 14 reference genomes identified by MEGAN, 11 were mapped along the majority of their genome with a high depth of coverage with the nr read set, demonstrating that similar phages were part of the Microgen cocktail. Microgen phages included three genera of the Myoviridae family: the genus Tevenvirinae with a 170-kb genome size (represented with the subgroups of T4-, RB69- and RB49, but not JS98-like phages Suppl. Fig. 2A and B), Felixounalikevirus (the broad host range *Salmonella* phage Felix O1 (Villegas et al., 2009; Whichard et al., 2010) with a 86-kb genome) (Whichard et al., 2010) (Suppl. Fig. 2E), and the newly proposed genus of rv5-like Myoviridae with a 140-kb genome (Santos et al., 2011; Schwarzer et al., 2012) (Suppl. Fig. 2D). Microgen phages also included three

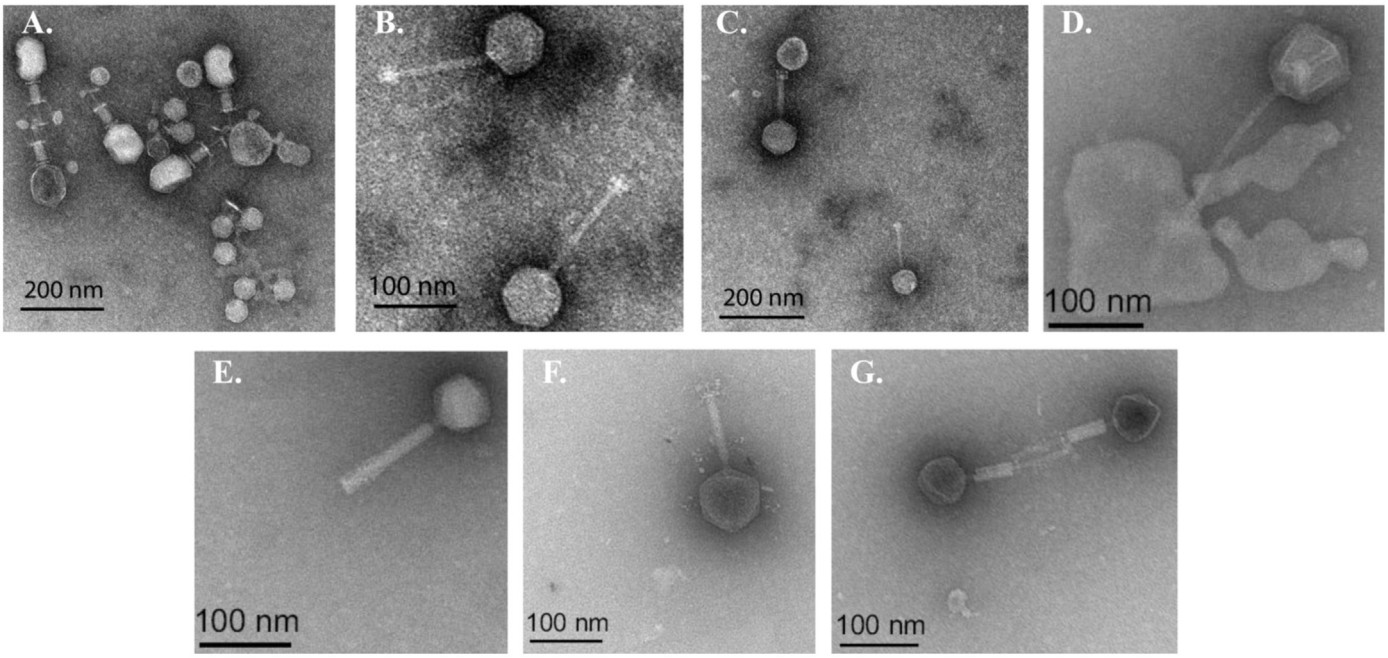

Fig. 1. *Negative stain electron microscopy of bacteriophages directly concentrated from the Microgen phage cocktail.* (A): T4-like Myoviridae, T7-like Podoviridae, and membranous debris representing a typical view of the sample. (B–G): Gallery of phages which were identified after searching the samples for less abundant viruses, which differed morphologically. For example, phages from panel B and F differ in baseplate structure, phages from panel C differ in head size, phages from panel D and E in tail structure. Panel G shows phages with contracted tails from an undefined Myovirus. The panels are not with the same magnification, size estimates are provided by the scales.

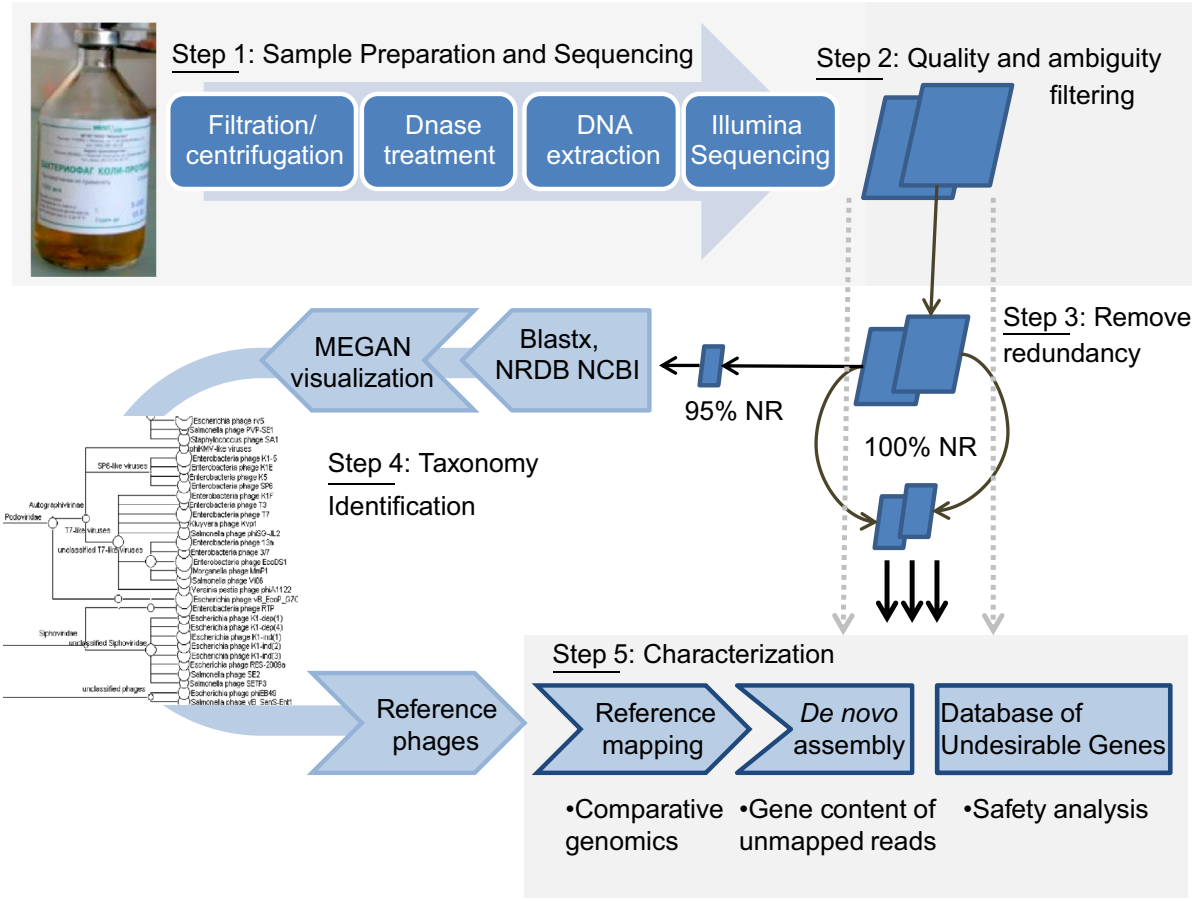

Fig. 2. *Metagenomic workflow.* During a first step, phages were sterile-filtered and concentrated by differential centrifugation to isolate phages. The phage pellet was then treated with DNase to remove free DNA not encapsulated into virions. Phage DNA was then extracted with phenol/chloroform and used for sequencing with Illumina technology. Then, the paired-end 100 bp reads were cleared from low quality and ambiguous sequences prior to the creation of read sets to be used for the later steps of analysis. Blastx searches of the 95% nr list were opened in MEGAN for taxonomic classification and identification of reference genomes to be mapped with the 100% nr read set. Reads not mapped to reference genomes were used for *de novo* assembly. Separately, the entire 100% nr read set was screened against the DUG database. Reference genomes and *de novo* assembled putative phage genomes, as well as DUG hits, were mapped again with the full read set to determine their relative abundance of phage DNA in the cocktail.

genera of the Podoviridae family: within the subfamily of Autographivirinae we identified the T7likevirus genus (with closest relatives to T7, K1F, and MmP1 phages, Suppl. Fig. 3A–C) (Scholl and Merril, 2005; Zhu et al., 2010) and SP6likevirus genus (closest relative $K_{1\text{-}5}$, Suppl. Fig. 3D) (Bull et al., 2010; Scholl et al., 2002, 2004) both in the 40-kb genome size range. The third Podovirus genus in the Microgen cocktail was N4likevirus (closest relative phage vB_EcoP_G7C, Suppl. Fig. 3E), which has a 73 kb genome with terminal repeats (Kulikov et al., 2012). A taxonomically still unclassified K1-ind(1)-like phage from the Siphoviridae family with a 46 kb genome was also part of the Microgen cocktail (Suppl. Fig. 4). The projection of the sequence reads on these reference genomes showed differences in depth of coverage (*e.g. T7* > *T4*). Small regions of no coverage were visible in many of these alignments in elements expected to show diversity, such as in the tail fiber region, endonuclease genes, and genes for hypothetical proteins. (Comeau et al., 2007; Miller et al., 2003; Tétart et al., 1998) Conflicts in the mappings represent the sequence variability of closely-related phages and indicate that each mapping was representative of several phages. (*e.g.* compare the left and right genome segments of T7, Suppl. Fig. 3A).

Bacterial DNA

Almost all "bacterial" matches in MEGAN showed upon manual re-investigation best hits to T7-like phages from *E. coli*. The few remaining "bacterial" hits were with putative prophage or mobile DNA elements from *E. coli, Shigella,* and *Proteus* integrated into bacterial chromosomes (matching integrases, transposases, helicases, portal proteins, terminase, or phage tail genes). The lack of sequence alignment of the nr reads with the reference genomes of *E. coli* NC_000913 and *Proteus mirabilis* AM942759, the assumed amplification strains for cocktail production, further confirmed that the phage fraction of the cocktail did not contain bacterial DNA. Exceptions were the *E. coli* gene *yfiD* involved in anaerobic glycolysis carried by a T4-like phage (Brüssow 2010; Sarker et al., 2012) and a *Proteus* gene annotated as a phage recombination protein (data not shown).

De novo assembly

Collectively, 61% of nr Microgen reads aligned with Caudovirales. No reads were shared between Myoviridae, Podoviridae, or Siphoviridae mappings. All reads attributed to the reference genomes identified in the MEGAN analysis at the set threshold were then removed from the nr set. This left us with 38% of nr reads, which were then used for *de novo* assembly (Fig. 3B).

From the set of the unattributed reads, 97% assembled into 1121 contigs, 508 which were larger than 500 bp length (Fig. 3B). Blastn searches revealed that 30 contigs had no hits in the

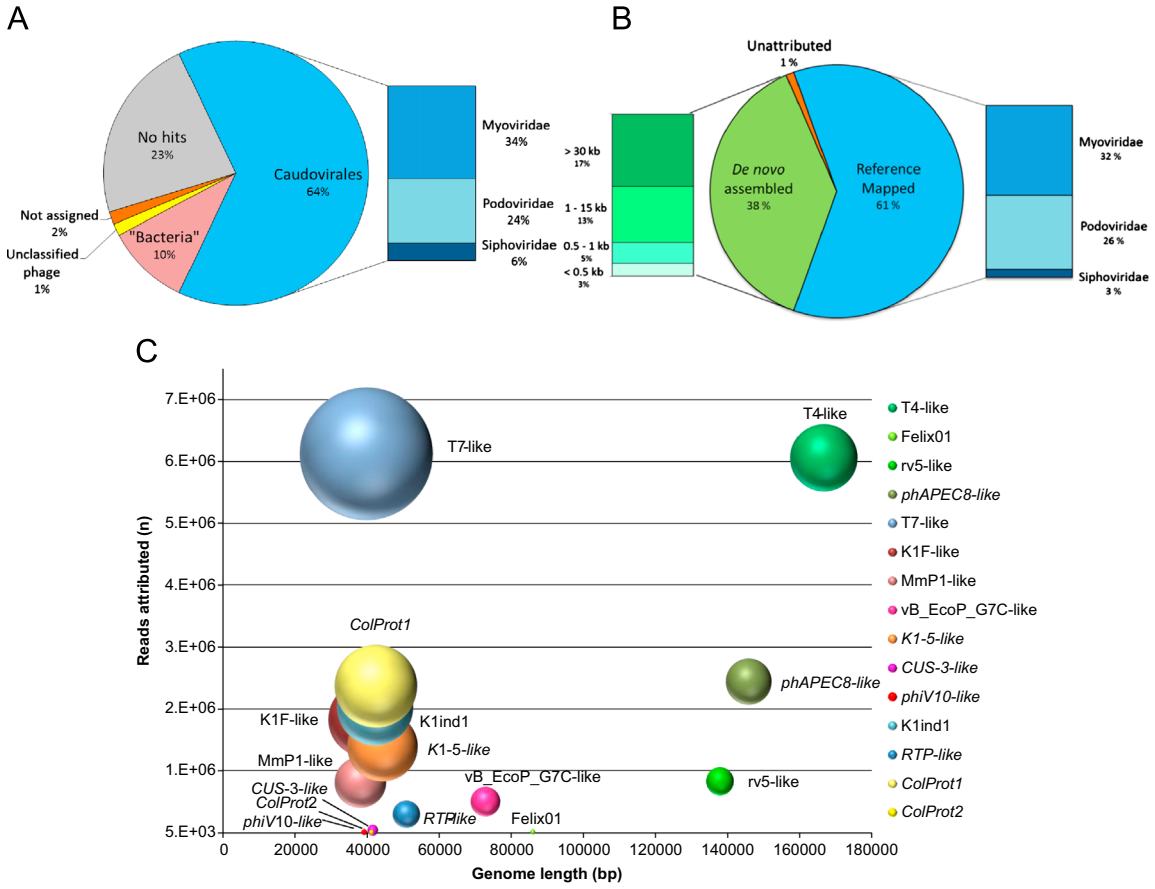

Fig. 3. *Statistics of Microgen phage cocktail sequence attribution.* (A). Proportion of best blastx match for 95% nr reads classified by MEGAN as Caudovirales (Myoviridae, Podoviridae, or Siphoviridae classified separately in the right expansion), "bacteria," unclassified phage, unassigned, or no hit. "Bacterial" hits were on manual re-analysis of phage origin (see Results), hence the quotation marks. Unassigned classification indicates that a hit was found for the read, but did not exceed the minimal threshold criteria for classification (default parameters). (B). Proportion of 100% nr reads attributed through reference mapping to known genomes (Myoviridae, Podoviridae, or Siphoviridae, right expansion), to *de novo* assembled contigs (separated by size of contigs, left expansion), or that remained unattributed. (C). Distribution of full reads to phages identified by reference mapping or *de novo* assembly (*de novo* contigs are indicated in italics). The size of the circles is proportional to the average coverage in reads of the genome. The circles are placed according to the genome size of the respective phage groups given on the abscissa and according to sequence abundance given on the ordinate in a logarithmic scale.

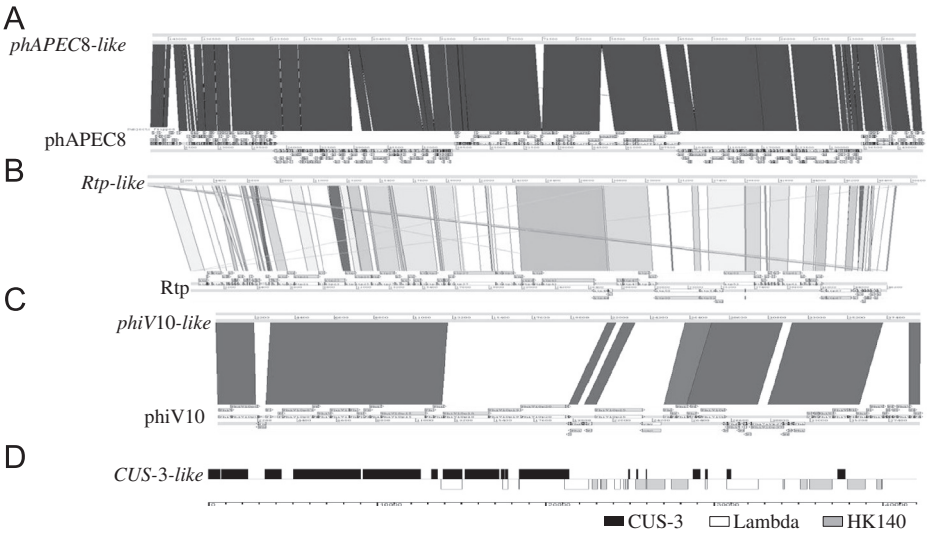

Fig. 4. *Selected de novo assembled contigs are aligned with related phage genomes*. Genome alignment of phAPEC8-like contig (A), the Rtp-like contig (B), and the phiV10-like contig (C) with their respective reference genomes. DNA sequence comparison is visualized with the Artemis Comparison Tool (ACT) and ORFs of the reference genomes are indicated by arrows. Homologous regions correspond to minimum blastn score of 100 and 80% identity. The shading gives a grading for the identity level of the aligned genome segments. (D). Regions of homology of a 40-kb contig with reference genomes CUS-3 (black), lambda (white), and HK140 (grey).

databases, while the remaining contigs had their best hits with phages from the Caudovirales order. The assembly also resulted in seven contigs greater than 30 kbp (Fig. 3B, Suppl. Fig. 5).

The largest of these contigs measured 147 kbp and it shared homology along 93% of its genome with the recently published phage phAPEC8, with an average nucleotide percent identity of

98%. Alignment gaps were over a tail fiber protein, a putative dTDP-glucose 4,6-dehydratase, and several hypothetical proteins (Fig. 4A). Phage phAPEC8 is a distant relative of the rv5-like Myovirus group (Tsonos et al., 2012) and was not yet present in the public databases at the time of our initial analysis and was thus not detected for reference mapping.

A contig of 51 kbp shared 69% of homology with phage Rtp (Fig. 4B), an unclassified member of the Tunalikevirus genus in the Siphoviridae family (Wietzorrek et al., 2006) whose 46-kb genome shares organization, but not sequence identity with *E. coli* phage T1. Comparison of the Rtp reference phage with this contig showed no or only partial homology for numerous hypothetical proteins, two endonuclease genes, a DNA primase, tail fiber, and a tail tape measure protein.

A contig of 39 kb shared similarity with the epsilon15-like Podovirus phiV10 over 63% of its genome length covering virion, lysis, lysogeny and replication genes (Perry et al., 2009) (Fig. 4C). This contig also aligned across 83% of its length with an unidentified prophage from *E. coli* strain IHE3034 (Accession number: CP001969).

A contig of 40 kb shared sequence identity over different regions with three temperate phages (Fig. 4D); namely the P22-like Podovirus CUS-3 (King et al., 2007) (over 47% of the contig length covering DNA replication, lysis and virion genes), phage lambda (integrase, antirepressor and CII protein) and phage HK140 (several hypothetical proteins).

Finally, two contigs of 40 and 42.5 kb length showed few matches. The first of which, ColProt1, shared protein sequence identity with phage DNA packaging and injection proteins, and the latter, ColProt2, shared only weak protein sequence identity over a quarter of its length with phage LIMElight (capsid, RNA polymerase, DNA polymerase) and phage KP3 (DNA maturase, DNA exonuclease).

Abundance of different phages in the cocktail

Through reference mapping and *de novo* assembly with the nr reads from the Microgen cocktail, we were able to identify the presence of 17 different groups of phages. However, it was necessary to analyze the full set of reads in order to gain insight into their relative proportions within the Microgen cocktail (anticipating no PCR amplification bias). This analysis identified T7-like phages as the most abundant ingredient of the Microgen phage cocktail, followed by T4-like phages, which was consistent with the EM observations. Nine further phage groups made important contributions to the cocktail that clustered, with the exception of two 140 kb- and one ~70 kb-long phages, around a genome size of 40 kb. Astonishing was the contribution of ColProt2: despite the fact that it showed no homology over 75% of its length it represents after T7-like phages, one of the most important contributors to the ColiProteus cocktail (Fig. 3C).

Undesired genes

We screened the Microgen reads against our in-house Database of Undesired Genes (DUG) (Zuber et al., 2007). Two relevant DUG matches were identified: one was to a gene conferring a greater environmental fitness to *E. coli* with respect to acid resistance (*dps* gene; Choi et al., 2000) and the other gene encoded a serum resistance factor (lambda *bor*-like gene (Barondess and Beckwith, 1995) from DLP12 prophage of the non-pathogenic *Escherichia fergusonii*).

Safety trial of Microgen phages in human volunteers

In a small safety trial we gave Microgen phages to five healthy adults as well as five, 5 to 10-year old children, and five children below 5 years of age, all from Bangladesh. The subjects received the phage at a high dose, at a ten-fold lower dose and placebo. Each subject received all three treatments in a random order and thus served as his/her own control allowing for the detection of adverse effects with a small group of exposed volunteers. The adults showed normal body–mass indices between 22 and 25, while children showed weight for age values in the mild sub-normal range (75 to 90%). These subjects received Microgen phages and placebo in random order. The subjects were followed by weekly complete physical examinations, which did not indicate problems (Table 1). Clinical chemistry analysis gave values outside of the normal range for serum creatinine and total serum proteins in adults and bilirubin mostly in children. Increased aspartate aminotransferase levels were observed in children younger than 5ys-old (Table 1). Hematology was mostly normal, however, eosinophils were elevated in 5 to 10 years-old children. Laboratory values outside the normal range were not more frequently observed when children were on high titer Microgen phage, as assessed by fecal phage count, than when on low phage regime and placebo (15 vs. 39 events); frequently these abnormal values were already observed in the subjects at the admission to the trial before any phage product was given (Table 1). Three adult subjects complained about transient abdominal pain, dyspepsia and tooth-ache, respectively. One of the older children reported ear pain, which was treated with antibiotics, while another reported an episode of cold. One of the younger children showed loose stool. None of these adverse events was associated with high dose phage application. No urtical rash or allergy was observed. No phage was detected in the blood stream nor was a serum antibody increase to phage seen in the study subjects (data not shown).

Fecal microbiota changes

Larger amounts of stool material were available for four adult volunteers to allow detailed stool microbiota analysis. The PCR products of bacterial 16S rDNA from the stool of the adult subjects were analyzed by denaturing gradient gel electrophoresis (DGGE) before and after application of the Microgen cocktail (Fig. 5A). Pair wise calculations using the Dice's similarity scores showed that profiles from the same subject were on average Dsc=86.8% similar over the three-week observation period. Visual inspection of the gels showed that the intensity of the bands varied over the observation period, suggestive of quantitative changes in fecal microbiota composition. This observation was confirmed by high throughput sequencing of PCR-amplified bacterial 16 S rDNA on serial stool samples of the adult volunteers (Fig. 5B). However, no consistent change in microbiota composition was seen in adult volunteers receiving the high phage dose when feces were three times sampled over a one-week observation period. Indeed, substantial changes in microbiota composition were observed in stool samples collected just two days apart. This short term variability in fecal microbiota composition was not induced by phage treatment since a similar variability in fecal microbiota composition was observed in adult volunteers when on placebo and sampled in an identical way (Fig. 5B).

Discussion

The Soviet phage therapy tradition has risen on one side hope, if not overt hype, for its potential as an alternative to antibiotics and on the other side skepticism, if not frank dismissal, as Stalinistic cure (Stone, 2002). Until the late 1930s Soviet phage therapy efforts were reported in the Russian literature in detailed publications much the same as those published in Western countries. The Second World War and the subsequent Cold War changed the situation, so that very few data were published on phage therapy and those that were published were extremely

Table 1
Characteristics of study subjects and observation of abnormal values in physical examination and clinical chemistry or hematology analyses in fifteen healthy volunteers from Bangladesh of different ages receiving Microgen phage cocktails and placebo.

Subject	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	Normal range
Characteristics of subjects:																
Age (y)	32	26	40	25	25	9.8	7.4	7.3	9.9	5.4	4.3	3.5	4.5	4	4.3	
Sex (1 = m)	1	1	1	1	1	2	2	2	1	2	2	1	2	1	2	
Weight (kg)	60	62	65	59	71	27	21	15	24	15	12	14	13	14	13	
BMI (1–5), Weight per age	*23*	*24*	*25*	*23*	*24*	85	91	64(1)	77	81	75	81	79	82	80	
Weight loss > 0.7 kg	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	< 0.7 kg (1.5%)
Pulse rate	–	–	–	–	–	–	a**3**	–	(1)	–	–	3	**3**	–	–	60–100/min
Respiration rate	–	–	–	–	**1**	–	–	–	–	–	–	–	a(2)	a(2)	–	20–30/min
Fever	2	–	–	–	–	–	–	–	3	–	–	a3	–	–	–	< 37.5 °C
Systolic blood pressure	–	2	–	–	a	–	–	–	a	–	–	–	–	–	a	110–140
Diastolic blood pressure	–	–	–	–	–	–	–	–	a	–	–	–	–	–	–	60–85
Clinical chemistry:																
Na^+	–	–	–	–	–	–	–	**1**	–	–	–	–	–	a**1**23	–	136–145 mEq/L
K^+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	3.5–5.1 mEq/L
Cl^-	–	–	–	–	–	–	–	**1**	–	–	–	a	–	–	–	98–107 mEq/L
Total CO_2	–	2	–	–	–	–	–	–	–	–	–	2	–	a	–	23–29 mEq/L
Ca^{2+}	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	2.1–2.6 mmol/L
Creatinine	–	–	a1	a12**3**	**1**23	–	–	–	–	–	a	–	–	–	–	20–100 μmol/L
Blood urea nitrogen	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	6–20 mg/dL
Total serum protein		a**1**23	a**2**	–	a2	–	–	–	–	–	–	–	–	–	–	6.0–8.3 g/dL
Albumin	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	3.4–5.4 g/dL
Total cholesterol		a2	a			–	–	–	–	–	–	–	–	–	–	< 5.18 mmol/L
Triglycerides	–	–	a	–	a	1	–	–	1	2	–	13	a12	–	–	< 1.7 mmol/L
Total bilirubin	–	a	–	–	–	**2**	a2	a**1**		2	a	a	a12	a	a**1**2	5.1–17 mmol/L
Alanine Aminotransferase	–	2	–	–	–	–	–	–	–	–	–	–	–	–	–	< 56 U/L
Aspartate Aminotransferase	–	–	–	–	–	–	–	–	–	–	a**1**23	2	2	a**1**23	a	< 40 U/L
γ-GlutamylAminotransferase	–	–	**2**	–	–	–	–	–	–	–	–	–	–	–	–	< 40 U/L
Fasting blood glucose	–	–	3	–	–	–	–	–	–	–	–	–	–	–	–	3.9–5.6 mmol/L
Hematology:																
Platelet count	–	–	–	–	–	–	–	–	**2**	2	–	–	a	3	–	170–500,000/μL
Red blood cell count	–	–	–	–	–	–	–	–	1	–	–	–	–	–	–	4.3–6.2 × 10^6/μL
Mean corpuscular volume	3	–	1	–	–	–	–	a**1**23	–	–				a**1**23	a**1**3	82–102 fL
Mean Corp. hemoglobin Conc.	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	31–35 g/dL
Hemoglobin	3	3	–	–	–	–	a1**3**	a**1**23	–	–	**1**2	1	2	a23	**1**	11–16 g/dL
Hematocrit	–	–	–	–	–	a	a1**3**	a**1**3	–	a**1**23			2	a**1**2	**1**	34–40%
White blood cell count	–	–	–	–	–	–	–	–	–	–	–	–	–	–	A**1**2	3.9–11 × 10^9/L
Neutrophils	–	–	–	–	–	–	–	–	13	–	–	a123	–	a	–	45–74% WBC
Lymphocytes	–	–	–	–	–	–	–	–	–	a	–	a123	–	a3	–	25–45% WBC
Monocytes	a	a	a3	**3**	–	–	–	–	a	–	–	2	–	**1**	–	3–7% WBC
Eosinophils	a**1**23	–	–	–	–	a**1**2**3**	a12**3**	a**1**3	3	a**1**2	23	a13	–	–	–	1–7% WBC
Adverse events	**1**(3)		1(4)	**3**	3(5)		2(6) **3**(7)	–	–	–	–	1(8)	–	–	–	

The first four rows give the physical characteristics (age in years, sex, weight in kg, and nutritional state as body-mass-index for adults and weight-for-age for children) of the 15 study subjects (columns 1–5: adults; 6–10: 5 to 10 y-old; 11–15: < 5 y-old children).
The next rows note individually for each subject if abnormal measurements were made during the physical examination, clinical chemistry or hematology analysis. "–" means that the volunteers showed only values that were within the normal range as defined with respect to the normal reference values given for children in the last column (for adults, the reference values given in Sarker et al. (2012) were used). Abnormal measurements were noted with "a, 1, 2 or 3" if they were observed at admission or after the first, second or third week of treatment. The abnormal value is underlined and given in bold when the volunteer received during that week the high titer Microgen phage preparation as assessed by fecal phage titration. Many normal subjects who were judged healthy for the local standard had "abnormal" values already at baseline, *e.g.* high eosinophil counts, which might reflect high exposure to parasites.
The next rows give in a similar way observation of clinical chemistry values and hematology values which are outside of the normal range.
Notes: (1) this child suffered from second degree malnutrition, (2) these children showed elevated respiration rates of 36/min, but chest examination was normal. Adverse events: (3): abdominal discomfort; (4): dyspepsia; (5): toothache; (6): ear pain; (7): antibiotics; (8): loose stool.

short. A large randomized double-blinded, placebo-controlled prevention trial of diarrheal diseases involving 30,000 children in the 1960s was, for example, published in a paper comprising 75 text lines and one table (Brüssow, 2005; Sulakvelidze et al., 2001). The Eliava Institute in Tbilisi has started to compile and publish old reports, but much material seems to be irretrievably lost (Brüssow, 2012).

Independent of these considerations, diverse phage cocktails against a wide range of infectious diseases are a registered medicine in Russia and sold in Russian pharmacies. The specter of a widening antibiotic resistance for human bacterial infections makes the exploration of phage therapy an important public health issue. Here we studied the phage composition of a widely sold phage product in Russia using two relatively rapid methods of characterization: electron microscopy and metagenome analysis. Biological approaches (such as isolation and characterization of phages) are time consuming and will underestimate the diversity as long as one does not know the production strains. Electron microscopy is quickly done and gives a relatively unbiased overview, and we could identify in the ColiProteus phage cocktail six distinct morphotypes, representing all three major groups of tailed phages. Due to their characteristic morphology, T4-like Myoviridae and T7-like Podoviridae were easily identified. EM however is not very sensitive, and only relatively abundant phages can be observed, and the relatively restricted morphological diversity of bacteriophages limits their classification. Metagenomics served as a broadening method to deepen our analysis of the cocktail composition.

Metagenome analysis has been widely used in environmental microbiology, mostly with respect to bacteria, but now also increasingly with respect to viruses (Reyes et al., 2012). This technique is also appropriate to characterize the viral composition

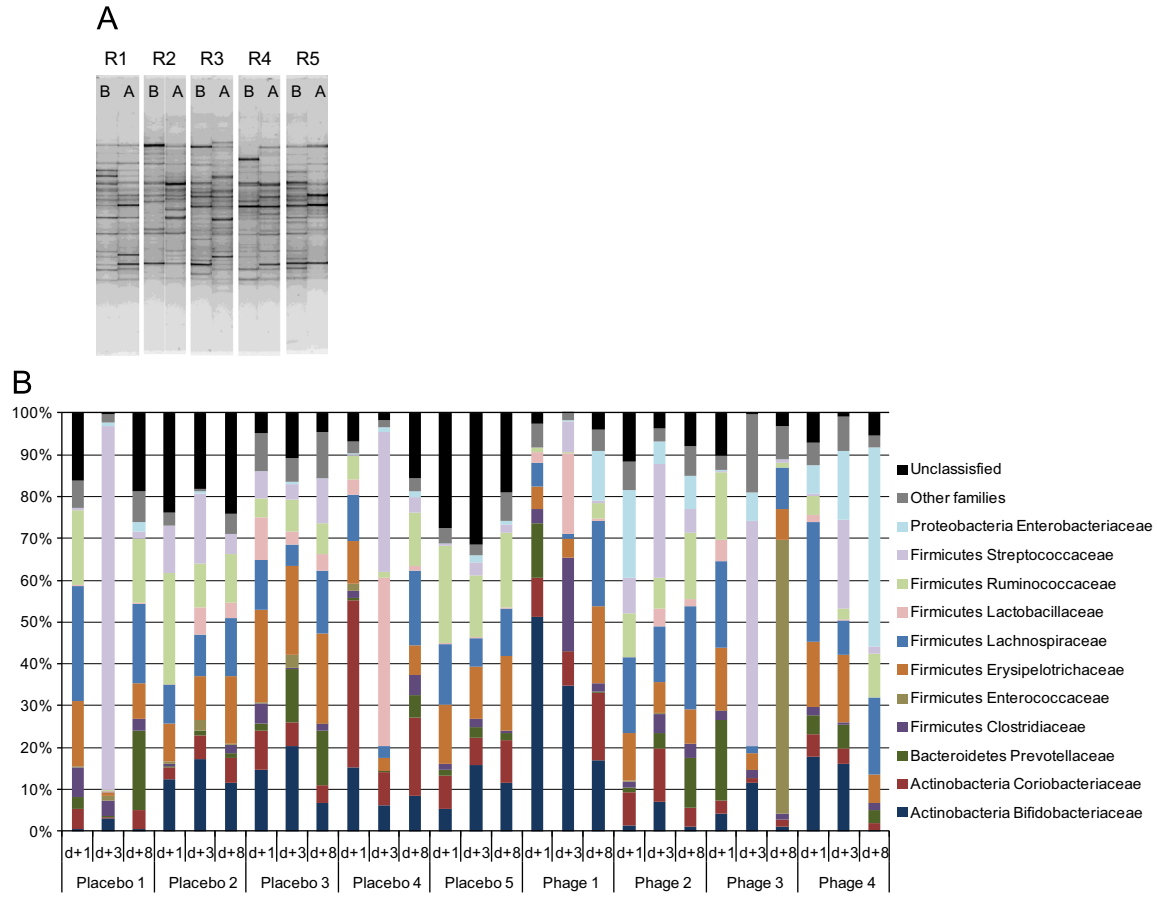

Fig. 5. *Fecal microbiota changes after phage application.* (A). DGGE profiles obtained with universal primers HDA1-GC and HDA2 targeting the variable 3 (V3) region of the 16 S rRNA gene in bacteria from the stool of 5 adults from Bangladesh at baseline (B, before receiving oral phages) and after receiving sequentially Microgen phages at high and lower dose and placebo, each for 2 days and separated by 5 days of wash out at the end of the 3-week follow-up (A, after receiving phages). (B). Bacterial community structure profiles for fecal samples from 4 adult volunteers receiving the Microgen phage cocktail (right) compared to 5 adult volunteers receiving placebo (left). The profiles are given for stool samples taken at days 1, 3, and 8 after oral phage consumption (right half of panel) or placebo (left half of panel) as specified on the *x*-axis. The serial stool samples of each individual subject are grouped next to each other and are separated from the next subject by a vertical bar. The panel gives the proportion of 16 S rRNA (in percent on the *y*-axis) amplified with universal primers targeting the variable 4 (V4) region of the bacterial 16S rRNA gene at the family level. The sequences attributed to a specific family are depicted in different colors, the color code is given at the right side. Only the eleven most prominent bacterial families are given, further minor families are lumped together as "other families". (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

of an unknown phage cocktail for which the industrial supplier cannot or does not want to provide its composition. Metagenomic analysis of the ColiProteus cocktail increased the number of different phage types that could be differentiated to 17. Not all of them were present with comparable amounts: consistent with the EM observation, T7-like, followed by T4-like phages were the major contributors. Other Podovirdae also made substantial contributions: two additional T7-like phage groups (K1F and MmP1), but also SP6-like, N4-like, P22-like and ε15-like genera of Podoviridae were identified. Beyond the T4 group, Myoviridae were also prominently represented in the cocktail with rv5- and phAPEC8-like phages, two recently described new Myovirus groups. Siphoviridae were also identified: distant relatives of the T1 phage group and an unclassified group of Siphoviridae ("K1ind1") were observed. To our surprise, the second most prevalent "phage" sequence after T7 was represented by ColProt1 obtained by *de novo* assembly. This contig presents a 42 kb genome typical for many phages, but has so few phage matches that its identity as a new phage is for the moment only tentative. Since *E. coli* phages are well investigated and now also well sequenced, one might suspect a *Proteus* phage behind this contig. No *Proteus* phage sequences are contained in public databases. In addition to these relatively major contributing phages, several minor phage groups were represented with much less sequence reads and low or no sequence conflicts suggesting that they were not intentionally added to the cocktail, but induced at low frequency from prophages contained in the hosts used for phage production.

Reference mapping and *de novo* assembly of the abundant phages showed diversity within each phage group, indicating that several related, but distinct phage isolates were contained in each group. This observation raises the question as to how Microgen is composing and propagating such a complex phage cocktail. A complex phage cocktail is not only difficult to produce and to maintain at comparable quality and reproducibility, but it also raises safety issues. We addressed the safety issue with two approaches, but both were limited in scope. The first was running the phage sequences against a database of undesired genes, where no matches of concern were found. However, here one might argue that this approach excludes only known virulence factors and undesirable genes, as the DUG database contains only genes for which a function has been assigned. Phage genomes frequently contain several new open reading frames (ORFs) for which function cannot be assigned that could code for undesirable functions not yet identified. This is a limitation to the safety aspect of phage genomes that should not be underestimated, even if the probability that these ORFs are "undesirable" is probably weak. Therefore, we set up a small safety trial to explore the reaction of healthy human volunteers to oral ingestion of the Microgen phage

cocktail, where the local ethical committee did not find evidence for adverse events associated with the consumption of this phage cocktail. Here, one might argue that the number of enrolled subjects was small and that rare side effects of the ColiProteus phage cocktail cannot be excluded with the present data.

Finally, one might also ask why Microgen opted for complex phage cocktails. That decision could reflect historical traditions of Soviet phage therapy experience where new phages were regularly added to the cocktail over decades to adapt it to the changing epidemiology of the targeted pathogens without the old phages being removed from the cocktail. The complex composition of the phage cocktail might additionally reflect the difficulty to get a good coverage of the pathogen. Most phages are not only species-specific, but they infect only a limited scope of strains from a given species. However, this coverage could theoretically also be achieved with a cocktail of phages from a single phage group, as we tested previously with a cocktail composed exclusively of T4-like phages (Sarker et al., 2012). The Microgen approach (or more generally, the Eastern European phage tradition) might also represent a hedge betting strategy. Our knowledge about the phage-bacterium interaction in the intestine is so fragmentary, even with the workhorses of molecular biology research such as phage T4 and *E. coli*, that we still do not know the factors governing the in vivo phage infection process (Maura et al., 2012a, 2012b; Brüssow, 2013). Microgen might have opted for including different groups of phages into the cocktail hoping that at least one group is efficiently replicating and lysing the *E. coli* pathogen in the gut. That this consideration is not a moot point is revealed by mouse experiments, where depending on the exact experimental conditions either T7-like (Weiss et al., 2009) or T4-like phages (Maura et al., 2012a, 2012b) are the better in vivo replicators. We hope to will get some answers on these questions and the virtues and drawbacks from different phage cocktail strategies by a side-by-side comparison of our T4-like and the Microgen ColiProteus phage cocktail in a controlled clinical trial in children hospitalized with microbiologocally confirmed *E. coli* diarrhea.

Material and methods

Phage cocktail

The commercial phage cocktail ColiProteus for the treatment of *E. coli* diarrhea was purchased from Federal State Unitary Company "Microgen Scientific Industrial Company for Immunobiological Medicines" of the Ministry of Health and Social Department of the Russian Federation (FSUC "SIC "Microgen", MOHSD RF). Export permits from Russia and import permits from Bangladesh were obtained. Authorization to test this product in subjects from Bangladesh was granted to the International Center for Diarrheal Diseases Research (icddr,b) at Dhaka/Bangladesh in the clinical protocol ICDDR, B #2008-062.

Microbiological tests

All laboratory methods (electron microscopy, fecal microbiota profiling and high throughput sequencing of the phage DNA isolated from the Microgen phage cocktail) were conducted as described previously (Sarker et al., 2012).

Illumina sequencing and MetaGenomic analysis

Samples were processed in vitro to generate a DNA template library of short inserts following a genomic shotgun protocol (Fasteris SA, Switzerland). High-throughput DNA sequencing was performed using HiSeq 2000 technology (Illumina) to produce 100 bp paired-end reads. Sequencing quality of the reads was verified using NGSqc Tool Kit (version 2.3) and sequences with a quality score < 20 were removed. In order to reduce the complexity, reads with a 100% sequence identity were removed from the total reads to generate a set of non-redundant (nr) reads.

This NR list was further reduced by removing reads that shared $> 95\%$ bp similarity. The closest homolog for these reads was determined using blastx searches for the best hit against the nr protein NCBI database. Reads with no hit were searched for their closet homolog by blastn searches against the EMBL phage database.

Taxonomical classification and reference mapping

The resulting blastx file was loaded into the MetaGenomic Analyzer (MEGAN) for taxonomical classification (min support= 100, min score=68). A representative reference phage genome from each taxonomical group identified in MEGAN was chosen based on dot-plot alignments (mummer package version 3.06 with default parameters, except that the minimum length of a maximal exact match was set to 20; the results were displayed with Mummerplot script with default parameters) and were used for reference mapping using (NC_000866, NC_005066, NC_004928, NC_005282, HQ829472, NC_011041, NC_007456, NC_001604, NC_011085, HQ259105, NC_008152, NC_007603, GU196279) against nr reads with SeqMan NGen software (version 3.1.0, DNAStar) (identity 80%, mer size 19). Mappings that covered the majority of the reference genome were further analyzed for coverage and sequence divergences.

De novo assembly

All reads that were not mapped to the reference genomes were compiled and *de novo* assembled with Seqman Ngen (identity 80%, mer size 19, DNAStar). Contigs were investigated for their closest homolog using blastn searches against the phage EMBL database (*e*-value≤0.001) and contigs larger than 10 kb were manually inspected. Results were visualized with Artemis Comparison Tool (ACT, version 12).

Safety screening

The list of NR reads was mapped against an in-house Database for Undesirable Genes (DUG), as described previously (Denou et al., 2009). Genes were identified as potential hits when at least one third of the subject gene was covered, and then these hits were re-mapped using the full reads set. Manual inspection of potential hits and homology searches were used to evaluate the safety implications from DUG hits.

Safety test

Fifteen healthy human subjects from Nandipara, Bangladesh were recruited for the safety study. Twenty mL of Microgen ColiProteus preparation in 150 mL mineral water (Vittel, pH: 7.3, 258 mg bicarbonate/L) was given three times per day to five adults over two days. For children the dose was reduced to 10 mL Microgen phage in 75 mL of mineral water given three times per day over two days (high dose). On *E. coli* indicator strain WG-5, widely used in ecology studies, we measured a titer of 7×10^{6} pfu per mL. This titer is, however, only a minimal estimate since *E. coli* phages not growing on WG-5 are not counted nor are *Proteus* phages considered. The same subjects also received Microgen phage at a ten-times lower dose (low phage) or placebo. Each subject received all three treatments in random order. The study

design was identical to that reported previously (Sarker et al., 2012). This type of design, known as repeated measures, uses internal controls and has thus no formal control group, *i.e.* subjects were studied when given placebo to represent internal subject-specific controls. Each product was given over two days and effects were observed over a week. Then a new treatment was given with again a follow-up for a week, after which the third treatment was given. This type of design presents some problems of analysis should delayed effects occur which might be attributed to the current product while it was in fact induced by the previous product. The 15 study subjects were followed over three weeks for adverse events by a panel of clinical, clinical chemistry and hematological parameters described previously (Sarker et al., 2012).

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2013.05.022.

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