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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

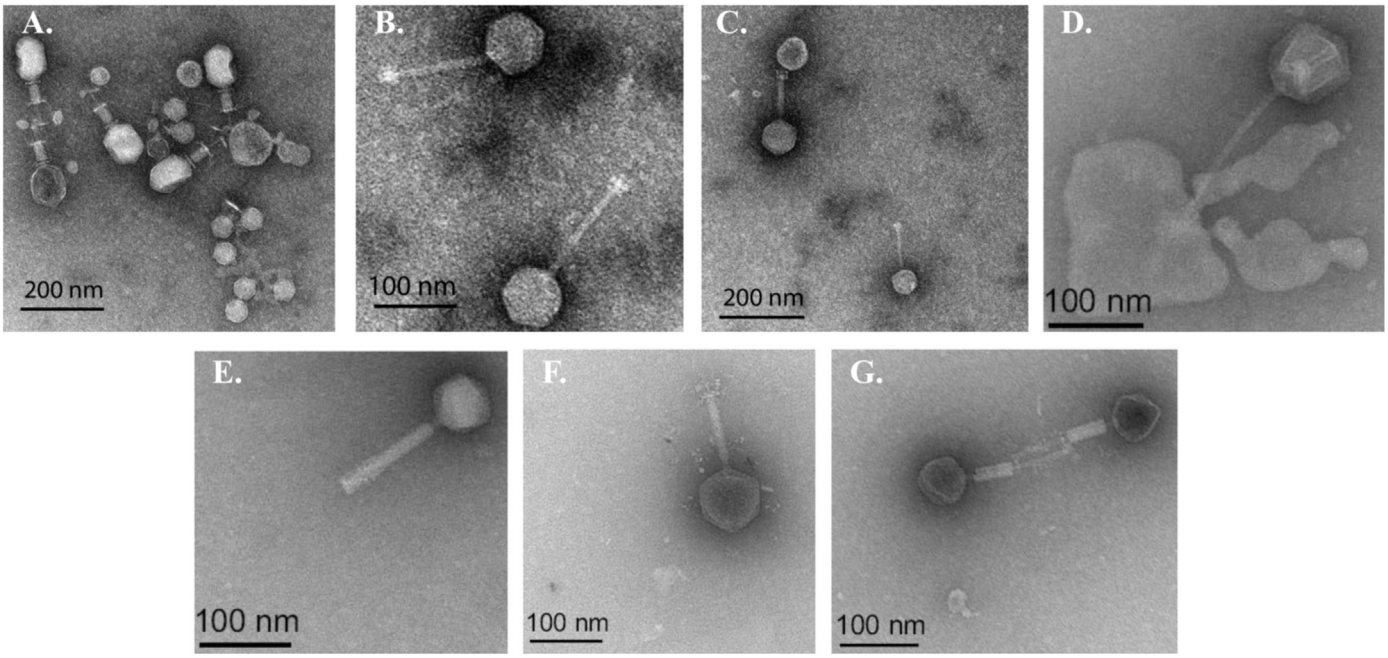

Fig. 1. *Negative stain electron microscopy of bacteriophages directly concentrated from the Microgen phage cocktail.* (A): T4-like Myoviridae, T7-like Podoviridae, and membranous debris representing a typical view of the sample. (B–G): Gallery of phages which were identified after searching the samples 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.