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A comparative study of different strategies for removal of endotoxins from bacteriophage preparations

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ABSTRACT

Bacterial endotoxins have high immunogenicity. Phage biology studies as well as therapeutic phage applications necessitate highly purified phage particles. In this study, we compared combinations of seven different endotoxin removal strategies and validated their endotoxin removal efficacy for five different phages (*i.e.* four *Pseudomonas aeruginosa* phages and one *Staphylococcus aureus* phage). These purification strategies included Endotrap HD column purification and/or CsCl density centrifugation in combination with Endotrap purification, followed by organic solvent (1-octanol), detergent (Triton X-100), enzymatic inactivation of the endotoxin using alkaline phosphatase and CIM monolytic anion exchange chromatography. We show that CsCl density purification of the *P. aeruginosa* phages, at an initial concentration of 10^{12}–10^{13} pfu/ml, led to the strongest reduction of endotoxins, with an endotoxin removal efficacy of up to 99%, whereas additional purification methods did not result in a complete removal of endotoxins from the phage preparations and only yielded an additional endotoxin removal efficacy of 23 to 99%, sometimes accompanied with strong losses in phage titer.

1. Introduction

The purification of bacteriophage particles is important for two reasons: either to investigate the phage particle on its own (*i.e.* phage biology studies) or for therapeutic application of phages, which is currently undergoing a resurgence (Adhya et al., 2014; Dabrowska et al., 2014; Ly-Chatain, 2014; Miedzybrodzki et al., 2012; Thiel, 2004; Vandenheuvel et al., 2015). When phages are propagated on Gram-negative bacterial hosts, endotoxins or lipopolysaccharides (LPS) have to be removed from these preparations. Endotoxins are part of the Gram-negative bacterial outer membranes and play an important role in the organization and stability of the bacterial cell (Ki et al., 1994). Bacterial endotoxins are well known for their immunogenic, pro-inflammatory and pyrogenic effects (Aderem and Ulevitch, 2000). In conditions where the body is exposed to endotoxins excessively or systemically, a systemic inflammatory reaction can occur, leading to multiple pathophysiological effects such as endotoxin shock, tissue injury and death (Anspach, 2001; Erridge et al., 2002; Ogikubo et al., 2004). Therefore, when phages are prepared for therapeutic purposes, it is crucial that different bacterial contaminants are removed which affect the efficacy and safety of the administration during phage therapy. The maximal level of endotoxins for intravenous applications of pharmaceutical and biological products is set at 5 endotoxin units (EU), *i.e.* 500 pg of endotoxins, per kg of body weight per hour (Daneshian et al., 2006). Additionally, bacterial endotoxins may also interfere with phage biology studies, especially when trying to establish the interaction of phages with the immune system.

Several strategies have been described for the removal of endotoxins from phage preparations. Here we compared different endotoxin removal strategies for the removal of endotoxins from five phages, *i.e.* four Gram-negative *Pseudomonas aeruginosa* phages and one Gram-positive *Staphylococcus aureus* phage (Table 1). The *S. aureus* phage forms a negative control for the endotoxin determination assay, as this phage is grown on a Gram-positive host which produces no endotoxins. Strategies were compared, taking into account the efficacy in removing endotoxins in relation to their effect on the phage titer yield.

2. Results and discussion

In this study, we evaluated the endotoxin removal efficacy of seven purification strategies (Fig. 1). To determine which strategy has the best endotoxin removal capacity, in combination with the minimal amount of phage loss, we calculated the 'endotoxin removal efficacy', defined as the ratio of the endotoxin units (EU) per plaque forming

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Table 1
Different phages used in the different purification strategies. For each phage, taxonomical family and bacterial host strain are presented, as well as the titer of the phage lysates, before application of any endotoxin removal techniques.

Bacteriophage name	Phage family	Bacterial Host	Titer (pfu/ml)	Reference	Isolated by	Isolation date
P. aeruginosa phage PNM	*Podoviridae*	*P. aeruginosa* strain 573	1.8×10^{13}	Merabishvili et al. (2009)	N. Lashki & M. Tediashvili	1999
P. aeruginosa phage LUZ19	*Podoviridae*	*P. aeruginosa* strain 573	5.0×10^{13}	Lammens et al. (2009)	P.J. Ceyssens	2006
P. aeruginosa phage GE-vB_Pae-Kakheti25	*Siphoviridae*	*P. aeruginosa* strain 573	2.5×10^{12}	Karumidze et al. (2012)	N. Kvatadze	2012
P. aeruginosa phage 14-1	*Myoviridae*	*P. aeruginosa* strain 573	3.6×10^{12}	Ceyssens et al. (2009)	V. Krylov	2000
S. aureus phage ISP	*Myoviridae*	*S. aureus* strain ATCC 6538	8.0×10^{13}	Vandersteegen et al. (2011)	Unknown	1920–1930

unit (pfu) multiplied by the phage recovery of the purified sample and the original sample subtracted from one (Table 2). The endotoxin quantification by Endozyme was validated by endotoxin quantification by means of Endosafe-PTS, for a selected number of samples. Both detection methods gave similar results within the same order of magnitude (Table S1)

The endotoxin removal strategies include either (1) Endotrap HD column purification alone (Merabishvili et al., 2009) (φET), or (2) CsCl density gradient ultracentrifugation alone (Lavigne et al., 2009) (φC) or (3) followed with Endotrap HD purification (φCET), and φET or φCET followed by either (4) organic solvent (1-octanol; Szermer-Olearnik and Boratyński, 2015) treatment (OS), (5) detergent Triton X-100 (Marcus and Prusky, 1987; Petsch and Anspach, 2000) treatment (TX), (6) enzymatic inactivation of the endotoxin using alkaline phosphatase (Bentala et al., 2002) (AP) or (7) anion-exchange chromatography (CIM DEAE disk column (CIM); Adriaenssens et al., 2012). We opted for these combined strategies, to compare the efficacy of purifying raw phage lysates (φET) *versus* CsCl-purified phages (φCET). As expected, phage ISP preparations from a Gram-positive host did not show any detectable endotoxin levels before or after any of the purification strategies (Table S1). The four *P. aeruginosa* phages contained between 326,000 and 7,465,000 EU/ml (Table S1). This concentration was

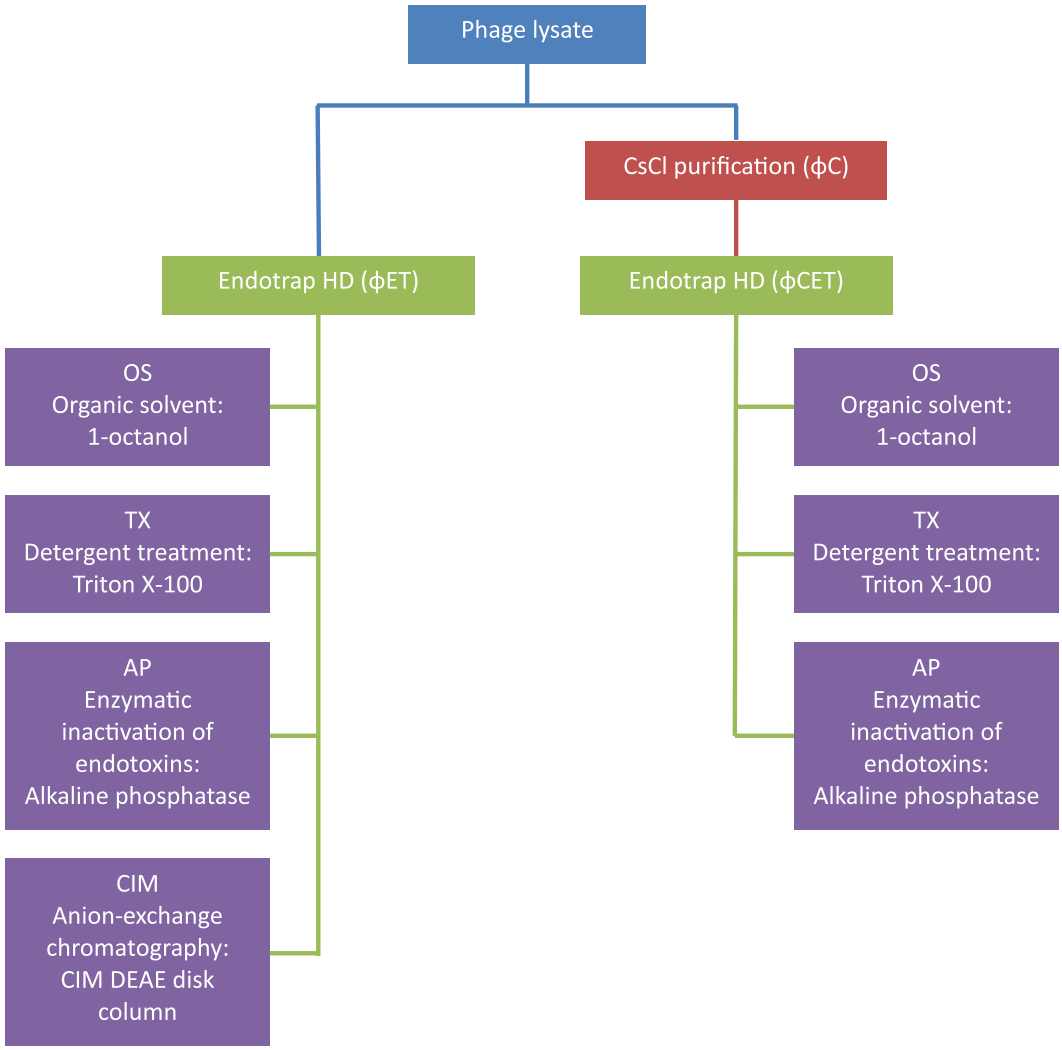

Fig. 1. Schematic representation of the different endotoxin strategies used, starting from different phage preparations. Phage lysates were obtained by the overlay-agar method. Part of this phage lysate was used either for (A) endotoxin removal using Endotrap HD (φET) or (B) further purified through CsCl density centrifugation followed by Endotrap HD (φCET). These preparations were further treated for the removal of endotoxins through different strategies: (OS) Organic solvent: 1-octanol; (TX) detergent treatment: Triton X-100; (AP) enzymatic inactivation of endotoxins: alkaline phosphatase; or (CIM) anion-exchange chromatography: CIM DEAE disk column (only performed on two phages).